Characterisation of Plasmodium Sexual Stage Antigens as Targets of Transmission Blocking Immunity

Thesis

Characterisation of *Plasmodium* Sexual Stage Antigens as Targets of Transmission-Blocking Immunity

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KEMRI-Wellcome Trust Research Programme, Kilifi, Kenya

Collaborating Establishment
Imperial College London, London, United Kingdom

Michelle Kathini Muthui
MSc, BSc

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Abstract

A promising strategy to achieve malaria elimination is by interrupting transmission using transmission-blocking vaccines (TBVs). TBVs target the sexual or mosquito stages of the malaria parasite to inhibit parasite development within the mosquito. To date, however, there are only three lead TBV candidate antigens Pfs230, Pfs48/45 and Pfs25 in various stages of clinical development. This project sought to identify novel antigens expressed on gametocytes, gametes and ookinetes with potential as TBV candidates. Furthermore, naturally acquired immune responses (NAI) to gametocytes have been described and have the potential to guide the development and the implementation of TBVs Therefore, this work also sought to improve our understanding of NAI to gametocytes. This was achieved by (1) carrying out a systematic review and meta-analysis of studies investigating NAI to the lead gametocyte-stage TBV candidates, and (2) assessing the changing patterns of gametocyte carriage at the Kenyan coast over time.

Key indicators of gametocytaemia and anti-gametocyte immunity were identified and evaluated against a novel panel of gametocyte antigens. These antigens, together with a separate set of gamete and ookinete stage antigens, were identified as potential TBV candidates using a combination of bioinformatic tools and laboratory investigations. Immunoprofiling of the identified gametocyte candidates provided evidence that stable responses can be generated to sexual stage antigens. Moreover, antigens that could serve as serological markers of recent gametocyte exposure, in particular PEB-P (PF3D7_0303900), were also identified. The ability of the sexual stage antigens to induce transmission-blocking immunity was also assessed. Promising candidates identified included PBCPP2 (PBANKA_0719100), a novel conserved and uncharacterised *P. berghei* protein), and SOAP (PBANKA_1037800). Further characterisation of these antigens may yield new candidates to add to the TBV development pipeline.

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List of Publications

Publications arising from work described herein:

1. Muthui MK, Kamau A, Bousema T, Blagborough AM, Bejon P, Kapulu MC. Immune responses to gametocyte antigens in a malaria-endemic population—the African *falciparum* context: A systematic review and meta-analysis. Front Immunol (2019) 10: doi:10.3389/fimmu.2019.02480 (**Chapter 2**)
2. Muthui MK, Mogeni P, Mwai K, Nyundo C, Macharia A, Williams TN, Nyangweso G, Wambua J, Mwanga D, Marsh K, *et al*. Gametocyte carriage in an era of changing malaria epidemiology: A 19-year analysis of a malaria longitudinal cohort. Wellcome Open Res (2019) 4:1–26. doi:10.12688/wellcomeopenres.15186.2 (**Chapter 3**)
3. Omondi, BR, Muthui MK, Muasya WI, Orindi B, Mwakubambanya SR, Bousema T, Drakeley C, Marsh K, Bejon P and Kapulu MC. Antibody responses to crude gametocyte extract predict *P. falciparum* gametocyte carriage in Kenya. Manuscript in preparation. (**Chapter 5**)

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List of Abbreviations

3D7	*Plasmodium falciparum* clone of parasite isolate NF54
AIC	Akaike Information Criterion
ACT	Artemisinin combination therapies
AMA1	Apical membrane antigen 1
AU	Antibody units
BSV	Blood stage vaccine
ChAd63	Chimpanzee Adenovirus serotype 63
ChAd63-MVA	Heterologous prime-boost regime of ChAd63 followed by MVA
CHMI	Controlled human malaria infection
CI	Confidence interval
CQ	Chloroquine
CSA	Chondroitin sulphate A
CSP	Circumsporozoite protein
DC	Dendritic cell
DDT	Dichloro-diphenyl-trichloroethane
DFA	Direct feeding assay
DMFA	Direct membrane feeding assay
ELISA	Enzyme-linked immunosorbent assay
G6PD	Glucose-6-phosphate dehydrogenase
GFP	Green fluorescent protein
GMS	Greater Mekong subregion
GPI	Glycosylphosphatidylinositol
HEK293E	Human embryonic kidney 293 Epstein Barr virus nuclear antigen 1 modified
IgG	Immunoglobulin G
IRS	Indoor residual sparing
ITN	Insecticide-treated net
IVOA	*In vitro* ookinete conversion assay
mAb	Monoclonal antibody
MDA	Mass drug administration
MPA	Murine population assay

MSAT	Mass screening and treatment
MVA	Modified vaccinia virus
NAI	Naturally acquired immunity
NASBA	Nucleic acid sequence-based amplification
NA-TBI	Naturally acquired transmission-blocking immunity
NK	Natural killer
PAM	Pregnancy-associated malaria
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEV	Pre-erythrocytic vaccine
PfDGFA	*Plasmodium falciparum* dual gamete formation assay
PfEMP1	*Plasmodium falciparum* erythrocyte membrane protein 1
PfPR2-10	*Plasmodium falciparum* parasite rate in 2 - 10-year olds
Pfs	*Plasmodium falciparum* sexual-stage antigen
PfSPZ	*Plasmodium falciparum* sporozoite
PH	Phenylhydrazine
PRR	Pattern recognition receptor
RBC	Red blood cell
RH	Reticulocyte-binding homologue
SMFA	Standard membrane feeding assay
SP	Sulphadoxine-Pyrimethamine
SP	Signal peptide
TBA	Transmission-blocking activity
TBI	Transmission-blocking immunity
TBV	Transmission-blocking vaccine
TEP-1	Thioester-containing protein 1
TLR	Toll-like receptor
TM	Transmembrane
tPA	Tissue plasminogen activator

Chapter 1
Introduction

1.1. Malaria

1.1.1. Disease burden

Despite concerted elimination efforts, malaria remains a significant public health concern in endemic countries where morbidity and mortality are greatest. The 2019 World Malaria Report estimated the number of malaria cases worldwide at 228 million cases (95% CI 206 – 258 million) in 2018[1]. Africa bears a disproportionate burden of the disease, accounting for 93% of the malaria cases, 99.7% of which are attributable to *Plasmodium falciparum*. Furthermore, while malaria-associated mortality declined from 533,000 to 380,000 between 2010 and 2018, the rate of decline slowed down from 2016 – 2018[1]. Stalling progress calls into question our ability to meet the Global Technical Strategy for Malaria 2016 – 2030 (GTS) goals for 2020. The malaria incidence rate for 2018 was estimated at 57 cases per 1000 population at risk; much higher than that required to meet the 2020 goal (45 per 1000)[2].

These data are not perfect, relying on reports from national malaria control programmes, household surveys of health service use or parasite prevalence to derive model-based estimates of malaria incidence[1,3,4]. Nonetheless, they provide a broad picture of the progress of malaria control efforts. Critical challenges faced by malaria control efforts include lack of adequate and consistent international and domestic funding, emerging parasite and vector resistance, and fragile health and surveillance systems[2]. For these reasons, malaria needs to remain a priority in national and global health agendas to avoid reversal of the gains made so far.

Malaria is caused by the protozoan parasite *Plasmodium* with the majority of deaths attributable to *P. falciparum* though *P. vivax* is also known to contribute significantly to malaria-associated morbidity and mortality in Southeast Asia and Latin America[5]. Other species known to infect humans include *P. malariae, P. ovale (P. ovale curtisi and P. ovale wallikeri)* though infection by either species is thought to be relatively mild[6,7]. The zoonotic *P. knowlesi* has also been shown to infect humans in South East Asia[8,9]. The bite of an infected female A*nopheles* mosquito transmits the parasite from

one host to another[10], making both parasite and vector targets for malaria control interventions. Control measures such as insecticide-treated nets and the highly effective artemisinin combination therapies (ACTs)[11–13] have proved invaluable in the fight against malaria and are discussed at length in section 1.3 below. Vaccines have been an important tool for the control and eradication of diseases such as smallpox[14,15], making them an ideal tool for the elimination of malaria. Currently, a licenced vaccine for malaria does not exist; however, pilot implementation studies on the RTS, S vaccine (discussed in section **1.3.** below) are currently ongoing in Kenya, Malawi and Ghana[16]. Despite the low and waning efficacy, the RTS, S vaccine paves the way for the development of more efficacious vaccines targeting infection, disease, and transmission to hasten us towards malaria elimination and eventual eradication.

1.1.2. Infection, disease and transmission

P. falciparum has a complex lifecycle requiring two hosts for replication and reproduction. The parasite cycles through 3 distinct stages (**Figure 1.1**): (1) parasite invasion and establishment of an infection within the vertebrate host mediated by a bite from an infected mosquito, (2) propagation of the parasite in erythrocytes leading to the clinical manifestations of disease, and finally (3) differentiation into transmissible forms that are taken up by a mosquito where they develop culminating in an infectious mosquito.

1.1.2.1. Infective stages

P. falciparum malaria infection in the human host begins when an infected mosquito bites a host injecting sporozoites into the dermis where they either remain in the dermis or migrate from the injection site via the bloodstream (~70%) or lymphatic vessels (~30%)[17]. Those that persist in the dermis or drain into the lymphatics are mostly degraded; however, a few partially develop within the lymph nodes into exoerythrocytic forms (EEFs). Those that enter the bloodstream traverse the sinusoidal barrier of the liver to reach the hepatocytes. The sporozoites then interact with hepatocytes via the thrombospondin repeat domain located at the C-terminal of the highly abundant circumsporozoite protein (CSP)[18]. Receptors on the hepatocyte such as scavenger receptor B1 (SR-B1) enable the sporozoite to penetrate the hepatocyte and form the parasitophorous vacuole (PV)[19]. Within the PV, the sporozoite develops into EEFs in the course of the next 2 – 10 days following the

mosquito bite, culminating in the formation of thousands of merozoites. The merozoites are then released into the bloodstream via budding of merosomes (vesicles filled with merozoites)[20].

1.1.2.2. Disease-causing stages

Once in the blood, the merozoites invade red blood cells (RBCs) in a rapid process to begin replication. First, the merozoite contacts the RBC and deforms the host cell. Two protein families mediate this process, the erythrocyte binding antigens (EBAs) and reticulocyte-binding homologues (RHs) that interact with RBC receptors such as the glycophorins A, B and C and complement receptor 1 (CR1) [21,22]. Interactions between members of these protein families and their receptors then trigger downstream invasion events, for instance, binding of EBA-175 to glycophorin A triggering the release of rhoptry contents[23]. Once initial contact is achieved, the merozoite reorients such that its apical end connects with the RBC membrane. The merozoite then attaches irreversibly to the RBC through the formation of a tight junction. Tight junction formation is aided by parasite-derived rhoptry neck protein 1 (RON1) complex and apical membrane antigen 1 (AMA1) interaction, triggering the expulsion of merozoite contents into the host RBC[24]. Upon RBC infection, the parasite develops over cycles of 48 hours, maturing into the ring stage, the trophozoite stage and finally a schizont filled with approximately 20 merozoites which are released upon schizont rupture to infect new erythrocytes[25]. These repetitive cycles of replication occur involving invasion, replication, egress and re-invasion contributing to the pathological features of malaria infection. The asexual forms cannot infect a mosquito, requiring the production of transmissible gametocytes in a process referred to as gametocytogenesis[26,27].

1.1.2.3. Transmission stages

A small proportion of the asexual parasites undergo commitment to sexual development. The AP2-G family of DNA-binding proteins that activate early gametocyte genes likely regulate this process[28,29]. Commitment can either occur within the same cycle of development or in the subsequent cycle upon schizont egress[30] with all resulting daughter merozoites going on to become either male (micro) or female (macro) gametocytes[31]. The decision to follow the sexual development pathway can occur spontaneously with a fraction of the asexual parasites committing

to gametocytogenesis[28]. Alternatively, gametocytogenesis may be triggered or amplified by host factors such immune pressure (reviewed in Talman *et al*. (2004)[32]) or parasite factors such as contents of extracellular vesicles released from infected erythrocytes[33,34]. Gametocytes develop through five distinct stages with stage I being morphologically similar to trophozoites; however, as they progress through stages II to V, they gradually adopt a distinct crescent shape[27]. Mature stage V gametocytes are the only stage found in peripheral circulation and are the transmissible stage with the immature gametocytes sequestered in the spleen or bone marrow[35]. While in the human host, stage V gametocytes are arrested at the G_0 phase of the cell cycle awaiting activation within the mosquito[31].

Once within the mosquito midgut, the changes in pH, drop in temperature, and presence of xanthurenic acid activate the female and male gametocytes causing them to differentiate into male and female gametes[26]. Within 20 minutes, male gametocytes undergo eight rounds of DNA replication, becoming motile flagellated microgametes in a process termed exflagellation. The female gametocytes round up and develop into macrogametes. During exflagellation, young microgametes adhere to erythrocytes forming exflagellation centres. Motile microgametes then leave the centres to locate and fertilise the macrogametes[36]. The gametes fuse to form a zygote which then develops into a motile ookinete that traverses the midgut and forms an oocyst[37]. Sporozoites develop within the oocyst which subsequently bursts to release them into the haemocoel. From the haemocoel, the sporozoites travel to the salivary glands awaiting transmission through the next blood meal.

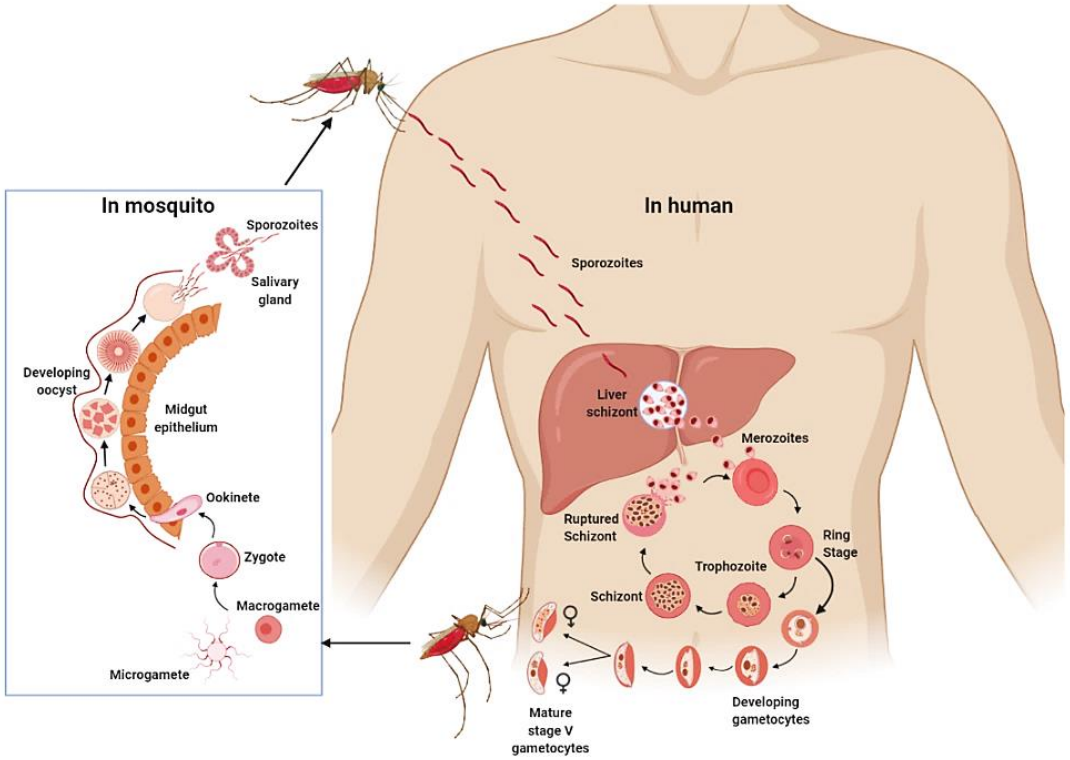


Figure 1.1: Lifecycle of *Plasmodium falciparum*. Diagram showing parasite development within the human and mosquito hosts. Image made using ©BioRender (https://app.biorender.com/)

1.2. Epidemiology of *Plasmodium falciparum* malaria

Plasmodium falciparum is widely distributed in the tropics and is responsible for 97% of the global malaria cases[1,38]. Furthermore, *P. falciparum*-malaria accounts for the majority of malaria-associated morbidity and mortality in Africa. While malaria is widely endemic in Africa, substantial heterogeneity in endemicity exists across the continent, with some regions experiencing stable transmission while others experience unstable or no transmission[39]. This is exemplified by the fact that ~80% of all cases occur in 18 countries, with the highest burden of disease being in Nigeria and the Democratic Republic of Congo[1]. Factors such as pre-existing *P. falciparum* burden, the suitability of vector habitats, as well as the varied implementation of control measures have led to convoluted spatiotemporal patterns of disease incidence and parasite prevalence. Malaria endemicity is directly related to transmission intensity, and therefore understanding the drivers of transmission is crucial to identifying at-risk populations and optimising intervention strategies[40]. Various metrics are used to define malaria transmission intensity across different populations. These include the entomological inoculation rate (EIR), parasite rate (PR) and seroconversion rate (SCR)[41].

The EIR is a measure of the number of infectious bites per person per unit time and is considered the 'gold standard'. However, the EIR technical and ethical considerations limit its utility. The PR measures the number of parasite-positive individuals in a region while the SCR relies on parasite biomarkers to determine the frequency with which malaria-exposed individuals seroconvert. Estimates of the PRs for different areas are easier to obtain and are more readily available across Africa[39] and can be used to categorise malaria endemicity into either (1) holoendemic regions where transmission occurs all year round, (2) hyperendemic regions where transmission is intense and seasonal, (3) mesoendemic regions where transmission is moderate, coinciding with seasonal epidemics, and (4) hypoendemic regions where transmission is lowest with a larger proportion of the population susceptible to disease[40]. Such classifications are essential for elimination efforts as they provide a means by which to prioritise, implement, and monitor malaria control interventions.

1.2.1. Clinical manifestations of disease

In malaria-naïve individuals, infection is symptomatic and presents non-specifically as fever, chills, headaches, muscle aches, and nausea[42]. If left untreated or if initial treatment is ineffective, disease can progress to severe malaria. The pathophysiology of severe malaria is complex, with several hallmarks of severity such as; anaemia (induced by various mechanisms such as the destruction of infected RBCs (iRBCs), increased splenic clearance of uninfected RBCs or impaired RBC production), respiratory distress following metabolic acidosis, cerebral malaria potentially linked to the sequestration of parasites in the microvasculature, and increased inflammation[25]. Disease progression and manifestation are affected by several factors which may be geographical and social, parasite-related or host-related.

1.2.1.1. Geographical and social factors

As mentioned above, malaria transmission intensity varies geographically. The intensity of transmission can influence the mean age of severity, whereby in areas of high transmission, young children present with severe disease[38,42] However, over time and after repeated exposure, they develop naturally acquired immunity (NAI) (further discussed in section **1.4.3.** below) that protects them from disease, and therefore older children and adults rarely suffer severe disease. However, as transmission intensity decreases, disease also commonly occurs in older children and adults owing to the slow acquisition of NAI[38]. Malaria transmission intensity also affects patterns of disease manifestation. Owing to intense parasite exposure in high transmission settings, children are most susceptible to severe anaemia due to hyperparasitaemia[38,43]. Conversely, in low to moderate transmission settings, cerebral malaria poses considerable risk to children as opposed to severe anaemia as its severity tends to increase with age[38,42,44]. Furthermore, as older children and adults are also susceptible, they also present with multi-organ failure, renal dysfunction and pulmonary oedema, which are rare in younger children[42].

Aside from transmission intensity, obstacles in access to quality health care can exacerbate disease burden and severity. Social factors like the area of residence (rural vs urban), household wealth as well as the level of education of the primary caregiver can impact treatment-seeking behaviour[45]. Additionally, political and social unrest,

human disasters as well as disease outbreaks affect the implementation of malaria interventions[2] leading to sustained transmission and malaria incidence.

1.2.1.2. Host Factors

Host immunity plays a vital role in determining disease severity. As indicated in 1.2.1.1 above, individuals living in malaria-endemic areas develop NAI with age and repeated parasite exposure that allows them to control disease and hence they either exhibit only mild symptoms or remain asymptomatic. The exception to this is pregnant women who once again become vulnerable to disease possibly related to parasite sequestration in the placenta[46] as well as gestation induced immunosuppression[47]. Malaria in pregnancy is associated with severe anaemia in mothers, preterm births, low birth weight, and neonatal death[48]. In areas of low transmission, the risk to pregnant women remains regardless of parity. In contrast, in areas of high transmission, the risk is more pronounced in primigravid women indicating a role for protection by NAI[49,50].

The high parasite transmission that occurred prior to the advent of control measures also had a hand in shaping host-parasite interactions that we observe today. The establishment of *P. falciparum* as a human parasite likely occurred within the last 10,000 years following divergence from a gorilla parasite[51–53] (and reviewed in Carter and Mendis (2002)[54]). The long association with the parasite has therefore allowed the selection and maintenance of genetic variants that confer protection against malaria in populations living in endemic regions. Genetic variants such as the haemoglobinopathies, RBC receptor polymorphisms, and RBC enzyme deficiencies can modulate disease severity by compromising the parasites ability to establish an infection (discussed further in **1.4.1**).

1.2.1.3. Parasite factors

During the establishment of infection, *P. falciparum* expresses various ligands that interact with host receptors to facilitate invasion as well as the expansion of the parasite population. One of the properties of *P. falciparum* that enhance its virulence is its ability to invade RBCs using multiple redundant pathways[22]. This is key to supporting the establishment of high parasitaemia and overcoming RBC receptor polymorphisms[25]. However, the parasite may invade RBCs deficient of specific receptors at reduced efficiency, which may attenuate virulence leading to less severe

disease. Moreover, the parasite can modify the iRBC surface to express parasite-specific receptors that mediate adhesion to host organs such as the brain, lung, liver, and placenta. Adhesion is primarily mediated by *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) encoded by the variant, *var,* multi-gene family.

PfEMP1 molecules contain multiple adhesive domains classed as either Duffy Binding-like (DBL) or cysteine-rich interdomain regions (CIDR)[25]. Multiple combinations of subtypes of the DBL and CIDR domains allow different PfEMP1 ligands to bind a host of receptors. Adhesive phenotypes are not homogenous, and parasites with different domain combinations will bind to different receptors affecting tissue localisation and pathogenesis. For instance, parasites that cause pregnancy-associated malaria (PAM) bind to placental chondroitin sulphate A (CSA) via VAR2CSA[46]. VAR2CSA does not contain the CIDR domains that would be required to bind to receptors such as endothelial protein C receptor (EPCR) implicated in severe childhood malaria[55]. Furthermore, the interaction between host immunity and PfEMP1 expression can modulate disease severity. Variants causing severe disease express a subset of PfEMP1 varaints[56], development of immunity to these variants can select for less virulent parasites in subsequent infections hence less severe disease[25].

1.2.2. Factors influencing parasite transmission

The spatial heterogeneity in malaria transmission intensity across Africa[39] indicates a need for locally relevant malaria control programmes. Therefore, understanding the determinants of transmission is relevant to malaria control efforts as it allows control programmes to adapt to changes in transmission patterns and aids the identification of populations that could benefit from the targeted application of interventions. As malaria transmission declines and intervention coverage increases, the residual transmission will tend to aggregate in hotspots[57,58] necessitating the focused implementation of control measures. Elimination programmes may then need to shift emphasis from tools to reduce morbidity and mortality, to tools that are suited to interrupting human to vector and vector to human transmission.

1.2.2.1. Ecological, geographical and social-economic factors

Environmental factors influence both mosquito and parasite development and survival, thus impacting malaria transmission. Furthermore, the suitability of habitats varies spatially and contributes to the observed heterogeneity in transmission across

regions and countries. Variables such as temperature, rainfall, vegetation density, and topography can impact malaria incidence. Temperature affects parasite and mosquito traits such as the length of sporogony, larval development, mosquito mortality[59,60] and consequently, malaria transmission. Work by Shah *et al*. (2019) across four different sites in Kenya, where *P. falciparum* is the main cause of malaria, proposed that an optimal temperature of around 25°C coincided with peak malaria transmission[61]. The authors also propose that with the threat of a warmer world owing to climate change, regions with cooler climates will move from seasonal epidemics to endemic transmission with the converse being true for regions with warmer climates. While the optimal temperatures for transmission may vary with mosquito and parasite species, there is a risk that climate change may threaten gains made in malaria control by placing new populations at risk of sustained malaria transmission.

Seasonal variations in rainfall also influence transmission, with peaks in malaria incidence typically following the onset of the rainy season. Several factors can contribute to this, including increased vegetation cover that provides shelter to mosquitoes[62], increased larval breeding sites[63] as well as increased host infectiousness to mosquitoes[58]. Proximity to suitable habitats has been linked to an increased risk of malaria[64,65] and provides a means to identify hotspots of active transmission for targeted interventions. Furthermore, factors such as homestead construction, a preference for cooking or sleeping outdoors as well as variations in patterns of bed net use may affect exposure to mosquitoes[58,64]. *Anopheles* species vary in feeding and resting behaviour, with *An. arabiensis* being more exophilic and exophagic in contrast to *An. coluzzi* that are endophilic and endophagic[58,66]. Therefore, social factors, as well as local vector composition, may make some communities or households more attractive to vectors, contributing to transmission hotspots.

1.2.2.2. Host-related factors

Human-mosquito transmission occurs when a mosquito takes up gametocytes during a blood meal. The capacity to infect mosquitoes is varied across individuals, being influenced by malaria transmission intensity and factors such as age, disease severity, and genetic background. In high transmission settings, children typically contribute significantly to the infectious reservoir owing to a higher parasite (and consequently gametocyte) burden as they are more likely to suffer acute disease. Indeed, in analyses

of gametocyte carriage, children under the age of five tend to have high gametocyte prevalence[67–70]. However, adjustments for demography and the likelihood of being bitten by mosquitoes increase the relative contribution of older children and adults to the infectious reservoir[71,72].

Furthermore, specific anti-gametocyte immunity may play a role in suppressing parasite transmission. Immune responses have been detected to the immature gametocyte infected red blood cell[73] as well as to the surface of the mature transmissible stages[74] (discussed further in section **1.5.3**). Responses to the immature stages may impact gametocyte density within the host[73]; however, this requires further investigation. On the other hand, responses to the mature stages impact parasite development within the mosquito affecting an individual's infectiousness[74].

Additionally, the physiological state within the host may also affect the production of gametocytes. Parasite conversion rate, the investment in gametocyte production compared to asexual parasites, varies during an infection[32,75]possibly in a bid to cope with infection-induced changes in the host environment[75,76]. The parasite must decide whether to invest in replication to maximise within-host survival or whether to invest in reproduction when survival in the host seems unlikely. Factors such as immune pressure, loss of red blood cells and drug pressure may modulate gametocyte production[32,77,78] by signalling worsening conditions within the host. As with disease severity, genetic traits that confer protection against malaria have been associated with an increase in gametocyte production. For instance, studies have found a link between sickle cell trait and an increased risk of gametocyte carriage [79–81]. Similarly, Grange *et al*. (2015) found that children of blood group O were associated with increased odds of being gametocyte carriers[68]. Again, this may stem from the fact that once the parasite detects a decreased likelihood of maintaining a successful infection, it may invest more in transmission to maximise survival.

1.2.2.3. Vector-related factors

Different vector species vary in their permissiveness to *P. falciparum* infection. Genetic differences can alter the mosquitoes innate immune response to infection. Mosquitoes can respond to parasite infection by encapsulating the developing ookinete in melanised structures, thus killing the parasite[82,83]. Melanisation contributes to the activation of components of the mosquito's innate complement-like

system such as thioester-containing protein 1 (TEP1), which also promotes parasite lysis[84]. Refractoriness to infection will decrease infectiousness to humans, thus limiting transmission, and hence this can be explored as a novel vector control tool. In addition to innate genetic resistance, co-infection of mosquitoes with microbial symbionts may affect the ability of the parasite to develop within the mosquito midgut. Herren *et al*. (2020) have shown that *Anopheles arabiensis* co-infection with microsporidia significantly impairs parasite transmission[85]. In addition to biological resistance, the vector species dominant in a region can influence local transmission intensity. An investigation into the factors sustaining transmission despite high intervention coverage in the Gambia by Mwesigwa *et al*. (2017) found a role for heterogeneous vector populations[58]. The authors found that the dominance of *An. arabiensis* and *An. coluzzii* in the eastern region contributed to higher transmission as these vectors are better adapted to the local ecological conditions.

1.2.2.4. Parasite-related factors

Parasite virulence factors (e.g., PfEMP1) that allow the parasite to evade the host's immune system and establish either acute or chronic infections (discussed in **1.2.1.3.** above) also indirectly facilitate transmission by increasing the parasite's chances of survival. Additionally, parasite ligands that bind to mosquito midgut receptors can also facilitate transmission. One example is the parasite protein Pfs47 that may aid the parasite's evasion of the mosquito's innate immune system by inhibiting TEP-1-mediated lysis[86]. Moreover, *Pfs47* exhibits extensive polymorphism with *Pfs47* haplotypes clustering geographically, possibly reflecting an adaptation to local mosquito species[87]. Only parasites expressing a compatible *Pfs47* allele can successfully evade the mosquito's immune system allowing parasite development and transmission. *Pfs47*-mediated immune evasion can thus be exploited to design interventions that block parasite transmission.

1.3. Efforts to eliminate malaria

Considerable reductions in disease burden have been observed over the years, attributable in part to the increased deployment of insecticide-treated nets (ITNs) and widespread use of artemisinin combination therapies (ACTs) as the first-line treatment for malaria[11–13]. No licensed vaccine exists for malaria, yet, however, it is recognised that an efficacious vaccine will be required if we are to achieve malaria

eradication. The current tools for malaria control, challenges faced in their implementation as well as efforts to develop novel interventions are discussed below.

1.3.1. Vector control

Vector control played a role in the elimination of malaria from the United States and Europe, being instrumental in controlling malaria in countries such as India and Sri Lanka in the 1950s – 1960s when dichloro-diphenyl-trichloroethane (DDT) was widely implemented in a global eradication programme[88,89]. However, the development of resistance, environmental concerns, and a lack of political and financial support led to the abandonment of the campaigns. Consequently, malaria persisted in endemic areas of Africa and was resurgent in countries such as Sri Lanka, Swaziland and Madagascar leading to deadly epidemics[89]. Current strategies to control malaria vectors have thus far relied on insecticide-treated nets (ITNs)[11,12,90], indoor residual spraying (IRS)[90,91] and larval source management[92]. While these interventions have had an impact on controlling vector populations, their success is limited by the development of insecticide resistance[93,94]. Insecticide resistance is particularly worrying in high-burden countries in West and Central Africa where parasites resistant to the various classes of insecticides have emerged. In addition to reduced susceptibility to insecticides, mosquitoes can also exhibit behavioural resistance by altering feeding and resting behaviour[94]. Therefore, while Long-lasting ITNs and IRS protect against mosquitoes that rest indoors and feed at night, they are ineffective against mosquitoes that feed in the early evening or those that predominantly rest and feed outdoors[2]. To combat resistance, researchers are developing biological methods for vector control. These include genetic manipulation of mosquitoes to impair vector capacity[95] and infecting mosquito populations with endosymbionts that interrupt parasite development within the mosquito (e.g. Microsporidia[85]). Vector control has had a substantial impact on reducing the burden of malaria; however, by combining vector control with other strategies to reduce human infectiousness, greater success can be achieved.

1.3.2. Drug Treatment

1.3.2.1. Malaria treatment

Antimalarials have been a vital tool in the control of malaria, however, their use is continually threatened by the emergence of drug-resistant parasites. The emergence

of drug resistance to quinine, chloroquine (CQ), sulphadoxine-pyrimethamine (SP) and atovaquone limits their use, leaving the more recently identified artemisinin as the current first line of treatment for malaria[96]. Combinations of the short-acting artemisinin with long-acting partner drugs form the basis of the artemisinin-based combination therapies (ACTs) currently in use. ACTs are highly effective at clearing asexual parasites[13,97] and reducing gametocyte carriage[13]. However, while ACTs act against early-stage gametocytes, they are not effective against mature stage V gametocytes (reviewed in Bousema and Drakeley (2011)[98]).

The only WHO-recommended and licenced drug with efficacy against mature gametocytes is primaquine (PQ)[99]. However, there is potential for haemolysis, particularly in glucose-6-phosphate dehydrogenase (G6PD) deficient individuals[100]. Despite this, a single low dose of PQ is considered safe for use in individuals with the prevalent African variant, G6PD A[101–103]. Nevertheless, even with effective treatment of malaria infections, post-treatment gametocyte carriage is commonly observed and can result in onward malaria transmission[104–106].

Drug resistance to artemisinin and the emergence of resistance to ACT partner drugs is well described in the Greater Mekong Subregion (GMS), with documented reports of reduced parasite clearance times in Bangladesh, Nigeria, and the Democratic Republic of Congo (reviewed in Ashley *et al*. (2014)[97]). This demonstrates a need for the discovery of new drugs and for the development of other control interventions to reduce both disease burden and parasite transmission. Work is ongoing to identify novel compounds targeting both the asexual and sexual parasite stages. For instance, the release of an antimalarial compound library by GlaxoSmithKline (GSK) has allowed the development of high throughput screens to identify novel drug candidates[107,108].

1.3.2.2. Mass drug administration (MDA) and mass screening and treatment (MSAT)

Over the past ten years, malaria elimination efforts have explored the use of mass administration of antimalarials to interrupt transmission or reduce morbidity and mortality[109]. These efforts include (1) mass drug administration (MDA) where the entire population in a given area is treated with antimalarials regardless of symptoms or concurrent parasitaemia, and (2) mass or focal screening and treatment (MSAT or

FSAT) where the population is first tested for parasitaemia or symptoms of malaria and treatment offered to only those who test positive. MDA is only effective in the short-term reduction of malaria incidence and prevalence[109,110], WHO advises implementation of MDA in regions where elimination is feasible, and where there is concomitant use of vector control and proper surveillance[109]. Additionally, MDA can be considered for use in epidemics where there is urgent need to reduce disease burden, and in emergencies, for instance, the combat of multidrug resistance in the GMS[109].

To support this, MDA trials conducted in Southeast Asia using dihydroartemisin-piperaquine (DP) and a single low dose of primaquine have demonstrated substantial decreases in malaria incidence and prevalence after implementation[111,112]. However, the success of MDA programmes does suffer from difficulties with adherence and intervention coverage, providing vital considerations for such programmes. Unlike MDA, MSAT efforts have been met with mixed success, with some studies showing little evidence of a lasting impact on malaria morbidity and mortality[113–115]. Challenges cited by these studies include lack of sensitive molecular diagnostics to detect subpatent parasitaemia, high re-infection rates, and as with MDA, inadequate coverage and adherence. Successful implementation of MSAT programmes may thus require the deployment of field-based molecular diagnostics, increased community engagement, and frequent follow-up campaigns to detect re-infections.

1.3.2.3. Mass drug administration (chemoprevention)

Pregnant women and young children bear the brunt of malaria in sub-Saharan Africa. For this reason, the WHO recommends intermittent preventive treatment in infants (IPTi) with SP for infants residing in areas of moderate to high transmission, intermittent preventive treatment in pregnant women (IPTp) with SP and seasonal malaria chemoprevention (SMC) with SP plus amodiaquine in children under five years in the Sahel subregion[1]. IPTp implementation has been associated with a 40% reduction in risk of moderate to severe anaemia and a 61% reduction in risk of parasitaemia for women in their first and second pregnancies, as well as a corresponding 27% reduction of the risk of low birthweight in infants[116]. Likewise, IPTi has shown evidence of reducing clinical malaria, anaemia, and hospital admissions in infants[117], while SMC had a significant impact on the incidence of

clinical malaria[118]. The success of these programmes, however, is threatened by the emergence of parasite resistance. Several polymorphisms in the dihydrofolate reductase (DHFR) and dihydropteroate synthase (DHPS) genes confer resistance to antifolate drugs such as SP[119]. The use of SP in these chemopreventative programmes may select for *Pfdhfr-dhps* mutants[120] and predispose individuals to treatment failure[121]. Another challenge for IPTp, IPTi, and SMC programmes is a limitation in coverage stemming from limited access to and delivery of health services[1]. Therefore, trials into new drug combinations to overcome drug resistance, as well as an increased commitment to the sustained implementation of these programmes, are necessary.

1.3.3. Vaccines

Historically, vaccines have been a critical tool for the control and eradication of diseases such as smallpox[14,15], making them an ideal tool for the elimination of malaria. The updated Malaria Vaccine Technology Roadmap, MVTR, (2013) by the WHO has set an ambitious goal to have a licenced vaccine against both *P. falciparum* and *P. vivax* malaria with an efficacy of at least 75% against clinical malaria suitable for use in endemic countries by 2030[122]. Additionally, it seeks to develop transmission-blocking vaccines amenable to mass administration to reduce malaria transmission. Currently, the only malaria vaccine that has progressed from clinical trials to pilot implementation is the RTS,S vaccine that targets the pre-erythrocytic CSP[16]. However, after over four years of follow-up, the vaccine showed an estimated 36% (95% CI: 31.8 – 40.5%) efficacy against clinical disease in young children aged 5-17 months who received four doses of vaccine, and 25.9% (95% CI: 19.9 – 31.5%) efficacy in infants aged 6 – 12 weeks[123]. The suboptimal efficacy demonstrated indicates the need for better vaccines with enhanced immunogenicity that can control disease and reduce transmission as well as the spread of drug-resistant parasites[124]. Consequently, this will require the identification of new vaccine targets. In particular, combination vaccines incorporating several antigens from different life stages could accelerate efforts to achieve malaria elimination and eventual eradication[125].

1.4. Immunity to malaria

Malaria immunity is a process by which individuals limit parasitaemia and control severe disease. This immunity can stem from (1) an innate resistance to the parasite conferred by host genetics, (2) from an immediate response initiated to inhibit the

parasite without prior pathogen exposure, or (3) can be acquired following a previous encounter with the parasite resulting in an enhanced response upon subsequent exposure. The innate and adaptive processes involved in NAI are described below.

1.4.1. Innate resistance to malaria

Malaria-induced selection pressure may explain the high frequencies of protective RBC variants in malaria-endemic areas[126]. The requirement of *P. falciparum* for haemoglobin during growth has driven the selection of polymorphisms in genes encoding the alpha, α, (*HBA1* and *HBA2*) and beta, β, (*HBB1* and *HBB2*) chains of the haemoglobin tetramer. Commonly described polymorphisms (**Table 1.1**) include the single nucleotide polymorphisms glutamate to valine at position 6 (Glu6Val - HbS), Gln6Lys (HbC) and Glu26Lys (HbE) in the beta chain as well as α-thalassaemia that results from deletions in *HBA* (reviewed in Taylor, Cerami and Fairhurst (2013)[127]). Heterozygosity for these variants has been associated with protection from severe malaria[128–130]. Proposed mechanisms of protection include reduced parasite invasion and growth[131], enhanced parasite clearance by both the innate and adaptive immune systems[132,133] and reduced cytoadherence of iRBCs[134,135]. Aside from the haemoglobinopathies, genetic variation associated with genes encoding RBC surface receptors that interact with parasite ligands can alter disease severity. For example, polymorphisms at the ABO glycosyltransferase locus that lead to the O blood group have been associated with protection from severe malaria[136]. Analysis suggests that parasites invading RBCs of the 'O' blood group are not able to efficiently form rosettes (clusters formed by the interaction of iRBCs with uninfected RBCs (uRBCs) to promote sequestration[137]) reducing their ability to sequester in organs[138,139]. Rosetting is also mediated by the complement receptor 1 (CR1) on the RBC surface. A study by Opi *et al*. (2018) in Kenya showed that alternate alleles of CR1 either protect against (*Sl2* allele) or predispose to cerebral malaria (*McC*)[140]. However, the protective effect of the *Sl2* allele is only evident in individuals of a normal α-thalassaemia genotype. Protection from malaria by up to 40% has also been seen with individuals possessing two hybrid glycophorin B/A genes, termed the Dantu blood group[141], further demonstrating the range of RBC structural variants that have arisen in malaria-exposed populations.

Table 1.1: Summary of the commonly observed haemoglobinopathies

Haemoglobinopathy	Epidemiology	Genotype	Molecular Pathology	Clinical Phenotype
α- thalassaemias*				
α^{+}-thalassaemia	Global	αα/α-		Asymptomatic; normal RBC morphology and quantity
α^{0}-thalassaemia	Global	αα/--		Mild anaemia
Haemoglobin H (HbH) disease	Global	α-/--	Accumulation and precipitation of unpaired β-chains (HbH) in RBC	Chronic haemolytic anaemia with accompanying hepatic, splenic, skeletal and metabolic sequelae; transfusion support required
Hydrops fetalis/ Hb Barts	Global	--/--	Accumulation of unpaired γ chains in utero forming Hb Barts that does not release oxygen to tissues	Fatal *ex utero*
β-thalassaemias				
Minor (heterozygosity)	Global	Reduced expression of one β-globin gene		Typically asymptomatic; normal haematocrit but low mean corpuscular volume

Haemoglobinopathy	Epidemiology	Genotype	Molecular Pathology	Clinical Phenotype
Major	Global	Reduced expression of both β globin gene	Accumulation of unpaired α-chains leading to oxidative damage to RBCs and RBC precursors	Severe anaemia and transfusion dependence
Haemoglobin S	Central, East, and West Africa; Arabian Peninsula; South Asia	Glu -> Val at position 6 of β-globin gene	Aggregation of deoxygenated HbS into polymers leading to RBC deformation to sickle shape, haemolysis and microvascular obstruction	Sickle cell disease, transfusion dependence and acute chest syndrome when homozygous (HbSS); asymptomatic when heterozygous (HbAS)
Haemoglobin C	West Africa, predominantly western Burkina Faso and northern Ghana	Glu -> Lys at position 6 of β-globin gene	Formation of hexagon-shaped HbC crystals	Mild haemolysis and anaemia when homozygous (HbCC); asymptomatic when homozygous (HbAC)
Haemoglobin E	Southeast Asia, predominantly on the Thailand, Laos and Cambodia border	Glu -> Lys at position 26 of β-globin gene	Mild reduction in β-globin production	Mild anaemia, microcytosis and hypochromia

*Four copies of α-globin genes (chromosome 16: genotype aa/aa) and two copies of β-globin genes (on chromosome 11) are present in the human genome; normal adult haemoglobin (HbAA) is a tetramer of two a-globin and two b-globin proteins. Table adapted from Taylor *et al*. (2013)[127].

Enzyme deficiencies also impact parasite development within the RBC. Polymorphisms in the G6PD and pyruvate kinase (PK) genes have also been described to modulate disease severity. G6PD plays an essential role in maintaining redox balance within the RBC in response to the oxidising environment created by haemoglobin degradation during parasite development[83]. In G6PD deficient individuals hampered parasite growth corresponds with protection from severe malaria. Similarly, PK-deficiency may also lead to reduced parasite growth, as well as increased phagocytosis, hence protection against clinical malaria[142]. In addition to enzyme deficiencies, polymorphisms in components of the host's immune system, such as cytokines and T-cell receptors, can potentially impact disease severity (reviewed in Kwiatowski (2005)[143]).

1.4.2. Innate immunity to malaria

Innate immune responses are critical in limiting parasite densities and are activated once parasitaemia crosses a threshold[144]. These responses are strain and species-independent and targeted to conserved *Plasmodium* molecules. Following parasite infection, components of the innate immune system such as dendritic cells (DC), macrophages, natural killer (NK) cells and γδ T cells are activated initiating a pro-inflammatory response that restricts parasite growth and stimulates the adaptive immune response[145]. *Plasmodium* pathogen-associated molecular patterns (PAMPs) such as glycosylphosphatidylinositol (GPI) anchors, haemozoin and immunostimulatory nucleic acid motifs interact with pattern recognition receptors (PRRs) on cells such as DCs and macrophages to stimulate the inflammatory response[146]. Downregulation of the pro-inflammatory response by cytokines such as IL-10 and transforming growth factor (TGF)-β is required to modulate disease[145], failure of which contributes to the pathologies of severe malaria such as dyserythropoesis[147]. In addition to inflammatory responses and activation of the adaptive response, the innate response also plays a role in the clearance of iRBCs via non-opsonic phagocytosis mediated by mononuclear phagocytes[148].

1.4.3. Naturally acquired immunity to malaria

Seroepidemiological studies in malaria exposed individuals and malaria challenge studies have helped shape our understanding of NAI to malaria. Over 100 years ago, Robert Koch observed that adults living in malaria-endemic areas were better able to

control disease in comparison to children and transmigrants[145,149–151]. Retrospective analyses of data from patients deliberately infected with *Plasmodium* to treat neurosyphilis indicated that patients were able to control disease upon re-infection, confirming Koch's findings [152,153]. These early experiments led to the characterisation of the critical features of NAI, being that NAI: (1) is effective after sustained exposure, (2) is, to a degree, species and stage-specific, and (3) is acquired dependent on the level of parasite exposure. NAI is imperfect as sterilising immunity is never achieved through natural parasite exposure. However, individuals can develop immunity to severe disease and resistance to parasitisation.

In areas of high transmission, parasite prevalence and risk of adverse outcomes following malaria exposure is highest in children and decreases with age[38]. Anti-disease immunity develops rapidly allowing children to modulate morbidity at seemingly high parasite densities. On the other hand, anti-parasite immunity, that confers protection against high-density parasitaemia, is slower to develop[38]. In areas of low transmission, the risk of disease is uniformly distributed among age groups, and disease severity correlates with parasite density[154]. Adults living in areas with intense transmission are thought to be protected owing to their cumulative exposure, while children remain susceptible due to their limited exposure. Cumulative exposure allows the host to generate responses to a broad range of clonally variant antigens restricting parasite virulence[25] and also allows the acquisition and maintenance of long-lived memory B cells[155–157]. The requirement for cumulative exposure may also explain why immunity to malaria is slow to develop. Declining transmission could, therefore, lead to a higher proportion of the population becoming susceptible to disease should resurgence occur. Indeed, studies in Senegal and Kenya have indicated an increased susceptibility of older children[158,159] and adults[159] to malaria as transmission declines, possibly as a result of reduced population exposure.

NAI may also be species-specific, whereby, for instance, infection by *P. falciparum* does not protect against *P. vivax* infection[160]. Conversely, NAI may not necessarily be strain specific. Allelic polymorphisms in genes encoding parasite antigens give rise to distinct variant antigens that differ between strains of a single species of *Plasmodium*[38]. Strain specificity is thought to contribute to the slow acquisition of NAI. Bull *et al*. (1998) suggested that anti-PfEMP1 antibodies are variant-specific, as children were less likely to recognise the PfEMP1 variant expressed during an active

infection than a heterologous variant[161]. However, from as early as the neurosyphilis studies, there was also evidence to suggest strain-transcending immunity. Individuals re-challenged with a heterologous strain experienced some level of protection, though this was typically lower than protection seen in those re-challenged with the homologous strain[152,160]. Therefore, both strain-specific and strain-transcending immunity have a role to play in NAI. The diversity of parasite strains in the field can prove a challenge for malaria vaccine design, necessitating the identification of antigens or epitopes that induce strain-transcending immunity.

1.5. Immune effector mechanisms of NAI as an aid to vaccine design

Early work showed that the passive transfer of purified gamma globulin from malaria-immune adults to children with severe malaria enabled the children to control parasitaemia[162]. Such classical studies lent credibility to the feasibility of developing a vaccine against malaria. Research is now ongoing to identify parasite targets capable of inducing a protective immune response. One of the challenges that remain is identifying immunological endpoints, so-called correlates of protection, that predict vaccine efficacy. Immunological correlates that are easy and quick to measure can facilitate vaccine trials reducing the complexity, sample size, and length of follow-up required[163]. Some of the challenges to identifying immune correlates of protection include: (1) the multi-stage lifecycle of the parasite, that results in different forms of immunity, and (2) the organ-specific localisation of immune responses that can result in an imperfect correlation with responses measured in peripheral blood[163]. Notwithstanding these challenges, a better understanding of what constitutes a protective response will facilitate vaccine design and assessment.

1.5.1. Pre-erythrocytic immunity

Sporozoite injection into the skin triggers the immune system stimulating humoral and cellular effectors. Anti-sporozoite antibodies can mediate protection by (1) blocking traversal from the dermis to liver[164], (2) inhibiting infection of hepatocytes[165,166], or (3) opsonic phagocytosis of sporozoites in concert with monocytes and macrophages[167]. Additionally, infected hepatocytes may also present antigens via MHC class I and II molecules inducing CD4+ and CD8+ T cell responses (reviewed in Radtke, Tse and Zavala (2014)[168] and Crispe (2015)[169]). Naturally acquired antibodies to the sporozoite do not seem to confer significant protection to

the host as sterilising immunity is never achieved even with high parasite exposure. However, there is some evidence to suggest that NAI to sporozoites may lead to delayed time to patency in malaria-exposed individuals[170].

Studies on the immunological response following RTS,S vaccination have not revealed a clear correlate of protection. However, anti-CSP antibodies likely play a significant role in protection which is potentiated by moderate induction of a CD4+ T cell response[171–173]. Unfortunately, the efficacy of the RTS, S vaccine wanes rapidly and precluding the acquisition of sterile immunity despite observed reductions in morbidity[174,175]. Conversely, following the development of whole sporozoite vaccines, high-level and long-lived immunity has been generated in malaria naïve individuals[176]. Based on preclinical studies in mice and non-human primates, vaccine-induced, liver-resident CD8+ T cell responses may contribute significantly to the observed protection[177,178]. However, this has been difficult to validate *in vivo* as responses are typically measured in peripheral blood[179]. Nevertheless, the identification of a mechanism by which sterile protection can be induced provides a means to design more efficacious vaccines.

1.5.2. Erythrocytic immunity

Early studies of NAI highlighted the role of antibodies in protecting against disease[180,181]. Seroepidemiological studies then highlighted important antibody targets that were associated with protection against malaria[182–184], with rodent studies providing insights into the mechanism of action of these antibodies[185]. The humoral response plays a dominant role in response to asexual stage parasites owing to the lack of MHC class I and II molecules on the iRBC surface to trigger a cellular response. Antibodies can target the merozoite or parasite antigens displayed on the surface of the iRBC. Antibodies to the merozoite can prevent infection in several ways such as (1) inhibiting invasion of and development in RBCs[185], (2) mediating complement-dependent lysis of the merozoite[186], (3) prevention of merozoite egress from schizonts[185], (4) mediating opsonic phagocytosis by macrophages[187], and (5) mediating the release of reactive oxygen species by neutrophils to kill the merozoite[188]. Antibodies to iRBC parasite antigens can act by inhibiting cytoadherence and rosetting, and also by mediating phagocytosis of iRBCs via macrophages (reviewed in Bull and Abdi (2016)[189] and Chan, Fowkes and Beeson

(2014)[190]). Additionally, there is a role for cellular responses in enhancing antibody production and in the production of pro-inflammatory cytokines to restrict parasite proliferation (reviewed in Chan, Fowkes and Beeson (2014)[190]). Nevertheless, the functional ability of antibodies as measured *in vitro* assays tends to correlate well with protection[188,191,192]. Therefore, the quality of the antibody response may be a good correlate of protection against the asexual stages.

1.5.3. Sexual stage immunity

Studies on natural and experimental infections in humans with sexual stages, as well as experimental infections of rodents, have provided insights into transmission-blocking immunity (TBI)[73,193–197]. Inhibition of gametocyte development within the human host has not been studied extensively. Some studies have suggested that responses to the gametocyte-infected erythrocyte (GIE) are directed to the mature stage V gametocyte[198,199]. Conversely, Dantzler *et al*. (2019) have suggested that immune responses to the developing immature gametocyte iRBC and not the mature GIE are responsible for reducing gametocyte carriage[73]. More research is required to ascertain the role of responses to the GIE in reducing host infectivity. Responses to gamete antigens exposed to the human immune system upon the destruction of mature circulating gametocytes and their role in TBI have been more widely studied[74,200–203]. As with erythrocytic immunity, TBI is mediated largely by antibodies that can function by (1) phagocytosis of immature gametocytes within the host[73], (2) mediation of complement-dependent lysis of gametes[204–206], and (3) prevention of zygote to ookinete[207] or ookinete to oocyst transition[208].

Antibodies, complement, and cytokines are taken up by the mosquito during a blood meal and remain active against the developing parasite (reviewed in Sinden (2015)[209]), though titre and activity of these immune components diminish with time. A limited number of studies have examined the role of cellular responses in inhibiting the development of the sexual stages. However, early work did show that passive transfer of CD4+ T cells from gamete-vaccinated mice to naïve mice reduced the infectivity of mice to mosquitoes[210]. Moreover, the cytokines TNF-α and IFN-γ have been described to mediate gametocyte destruction within the host[211,212]. Nonetheless, the predominant mechanism of TBI seems to be mediated by antibodies. Assays aimed at assessing TBV candidate efficacy indicate that the quantity and quality of antibody response induced are reliable correlates of inhibitory activity. Antibody titre, avidity, and IgG subclass ratio correlate with the level

of inhibition observed[213]. Specifically, antibody titre seems critical to successful transmission-blocking activity (TBA). While high titres are associated with inhibition, low titres can lead to enhanced transmission (TE)[201,205,214,215]. When titres are low, antibodies binding to targets on female and male gametes may (1) promote their interaction within the midgut facilitating fertilisation, (2) induce conformational changes to surface molecules on the gametes to enhance interaction or on the zygote to stimulate development, or (3) protect the parasite from the mosquito's immune response[216]. The potential for TE is, therefore, an essential consideration for TBV development.

Naturally acquired antibodies to gametocyte/gamete surface antigens Pfs230[201,217–219], Pfs48/45[201,217–219], Pfs47[220] and HAP2[221] (discussed in **1.6.3.1.**) have been detected in individuals living in malaria-endemic areas. These antibodies are associated with TBA, and for Pfs230 and Pfs48/45, antibody titres correlate with the level of TBA observed[222]. Antibodies to sexual stage antigens are thought to be short-lived, depending on recent gametocyte exposure[217,218]. The short-lived nature of sexual stage responses may be explained by the induction of a relatively T cell-independent immune response[223,224]. This may relate either to the generation of responses to non-protein targets or to ineffective antigen processing that fails to stimulate CD4 helper T cells[223,224]. However, experimental infections in animal models have indicated induction of cellular responses[210–212] and also boosting of vaccine-induced responses with parasite challenge[225,226]. This evidence suggests that it is possible to generate some level of immune memory to sexual stage antigens. Seroepidemiological studies on the few identified transmission-blocking immunogens identified so far do not give a clear picture of TBI. For instance, some studies have shown no age-dependent increase in responses[217,218], while others have shown an age-dependent increase[203,227,228]. Further investigations into the nature of naturally acquired TBI are therefore warranted as they may aid TBV design.

1.6. Malaria vaccines

Malaria vaccines can be grouped into three classes, depending on their stage-specific targets[5,15,229]. Pre-erythrocytic vaccines target the sporozoite and seek to prevent clinical illness by inhibiting the development of infection, e.g. RTS,S[123], or the growth of parasites within the liver, e.g. multi-epitope thrombospondin-related adhesive protein (ME-TRAP)[230]. Asexual blood-stage vaccines seek to reduce parasite densities and subsequently reduce disease pathology, e.g. merozoite surface protein

(MSP)-1_{19}[90]. Finally, vaccines to interrupt malaria transmission, which includes transmission-blocking vaccines (TBVs) that target the transmissible stages of the parasite[122,231], e.g. Pfs25[90]. Additionally, mosquito antigens involved in parasite development within mosquito midguts have potential as TBVs, e.g. *Anopheles* alanyl aminopeptidase N 1 (AnAPN1)[232]. A summary of the different malaria vaccines in clinical trials is provided in **Figure 1.2,** and a description of the various candidates with insights into considerations for further development follows below.

1.6.1. Pre-erythrocytic vaccines

The most advanced malaria vaccine to date, RTS,S, is a pre-erythrocytic vaccine (PEV) targeted to the dominant sporozoite protein CSP. RTS,S is a subunit vaccine consisting of multiple copies of the central NANP repeat and C-terminal domain of the CSP protein (designated RT) fused to the hepatitis B surface antigen (HBsAg), with an extra HBsAg (designated S, S)[233]. The vaccine is designed to contain both B- and T cell epitopes[234,235]. However, protection seems to correlate predominantly with short-lived antibody responses, contributing to the low efficacy observed[174,175]. The challenge, therefore, for future CSP-based vaccines is to develop constructs or delivery platforms that induce long-lasting and high-titre antibodies. This could be achieved by altering the dosing regimen, targeting the full-length protein to include more epitopes, or by using more potent adjuvants for vaccine delivery[236]. Indeed, a next-generation vaccine in pre-clinical development, R21, contains a single copy of HBsAg rather than the four-times HBsAg excess present in RTS, S[237]. R21 appears to be more immunogenic in mice, possibly due to the greater amount of CSP displayed in comparison to RTS, S.

As an alternative to subunit vaccines that have limited epitope presentation, whole sporozoite vaccines (WSV) prepared using live-attenuated sporozoites are in clinical development. Seminal work by Nussenzweig *et al*. (1967) showed that injection of mice with gamma-irradiated sporozoites generated sterile immunity, proving the utility of this approach[238]. Radiation attenuated sporozoites (RAS) form the basis of the *Plasmodium falciparum* sporozoite (PfSPZ) vaccine that has been evaluated in controlled human malaria infection (CHMI) studies[176,179,239–242]. Trials in malaria naïve individuals have shown PfSPZ to be highly efficacious. However, the efficacy appears lower in adults from malaria-endemic areas, possibly due to immune

tolerance owing to chronic malaria exposure[197,241,242]. Therefore, considerations will have to be made on dose and regimen to enhance efficacy in endemic populations. In addition to RAS, genetically attenuated parasites (GAP) are in development where genes upregulated in sporozoites are deleted[243]. This allows developmental arrest much later than with RAS, thus enabling increased sporozoite biomass and presentation of a diverse array of antigens for an enhanced immune response. Triple deletion GAPs have shown promise in inducing sterile protection in rodent[244] and human studies[245] supporting their further investigation in clinical trials. An alternate approach to attenuation is vaccination with live sporozoites under drug cover, in particular chloroquine, PfSPZ-CVac. While this approach has shown promise in naïve volunteers, in terms of protective efficacy and breadth of responses generated[246], the use of live parasites will likely require additional safety considerations[236].

PEVs also target the liver stage. Currently, Thrombospondin Related Adhesion Protein (TRAP) fused to a multiple epitope (ME), or ME-TRAP is the most widely evaluated liver stage vaccine. ME-TRAP has undergone substantial optimisation, and the current formulation in recombinant replication-deficient viral vectors (ChAd63 ME-TRAP and MVA ME-TRAP) has been shown to induce potent T cell responses (reviewed in McCall, Kremsner and Mordmuller (2018)[163] and Ewer *et al*. (2015)[247]). Variable efficacy has been observed in adults and children[248–251], highlighting the need for further optimisation of vaccine constructs and vaccination regimen. The proteins expressed in the liver stage are not yet well defined, complicating the identification of novel targets. Despite this, two new antigens, liver-stage antigen 1 (LSA1) and liver-stage associated protein 2 (LSAP2), capable of inducing strong CD8+ T cell responses were identified[252], and are currently in Phase I/IIa clinical trials (ChAdOx1-MVA-LS2)[236].

1.6.2. Blood-stage vaccines

Identification of blood stage vaccine (BSVs) targets has been guided mainly by identifying merozoite antigens that are dominant targets of natural responses, or by rodent studies using monoclonal antibodies in invasion inhibition assays[253]. Through these approaches, vaccine candidates such as AMA1, MSP1, MSP3 and GLURP were identified. Clinical trials of blood-stage vaccine candidates have had mixed success, with several targets showing low efficacy. A Phase I/IIb trial of the Combination B

vaccine, consisting of two merozoite surface proteins (MSP1 and MSP2) and ring-infected erythrocyte surface antigen (RESA), showed some efficacy in reducing parasite burden but none against morbidity[254]. Similarly, vaccines based on AMA1[255,256] or MSP1[175,257] have shown low efficacy in preventing clinical malaria in endemic populations. Furthermore, the vaccine candidate GMZ2 consisting of the relatively conserved N-terminal of the GLURP protein fused to the C-terminal of MSP-3 showed only 14% efficacy against clinical disease in a large multicentre phase IIb trial[258].

BSVs based on immunodominant merozoite antigens such as AMA1 or MSP-1 face the seemingly insurmountable hurdle of antigen polymorphism. Extensive polymorphism may reduce their efficacy in field settings where diverse parasite strains circulate[254,256]. Furthermore, as merozoite invasion is accomplished in under one minute, antibody titres need to be high and of good quality (high affinity, avidity, and multiple epitope recognition) to have an impact[259]. To overcome antigen diversity, improved formulations, including a cocktail of alleles, e.g. AMA1 diversity covering vaccine (AMA1-DiCo)[260], are being investigated. Additionally, following on the improved efficacy seen with WSVs, whole blood stage vaccines (WBSVs) are being developed. These WBSVs use liposomal adjuvant antigen delivery platforms to avoid induction of alloantibodies to the RBC[261] or by genetic attenuation of blood stage parasites[262]. WBSVs have the advantage of presenting a diverse array of antigens and inducing strong T cell responses (reviewed in Wlison *et al*. (2019)[263] and Good and Stanisic (2020)[264]) and may overcome challenges faced by subunit vaccines.

Interestingly, the conserved merozoite invasion antigen reticulocyte-binding homologue 5 (RH5) has been shown to induce potent, strain-transcending immune responses despite the lack of a significant natural immune response[265]. This suggests that NAI may not always provide a reliable means for antigen selection. RH5 is an attractive candidate antigen as it is conserved among different parasite strains and mediates an essential interaction for merozoite invasion via its receptor, Basigin[266]. Studies in rodent[267] and simian[268] models, as well as pre-clinical trials in humans[269], are promising, and clinical trials using various antigen delivery platforms are underway (ChAd63-MVA-RH5 and RH5.1/AS01)[236].

Vaccines targeted to the iRBC have also been challenging to develop due to the extensive polymorphism of antigenic targets such as the PfEMP1 molecules. Aside

from being clonally variant, PfEMP1 molecules can undergo recombination further increasing the number of variants that could exist[189,270]. However, as functional constraint restricts parasite diversity and key *var* subsets are associated with severe disease, crucial molecules or epitopes can potentially be identified for vaccine design[189]. In support of this assertion, the conserved VAR2CSA implicated in PAM is currently under investigation as a vaccine candidate, with two candidate antigens in clinical trials (PRIMVAC[271] and PAMVAC[272]). A vaccine against cerebral malaria (CM) may also be in the pipeline following the identification of a PfEMP1 motif associated with the development of CM[273]. Though such a vaccine may not provide sterile immunity, it would reduce disease severity.

1.6.3. Transmission-blocking vaccines (TBVs)

The decline of malaria burden across the globe has made malaria elimination a feasible goal in countries where transmission has been declining steadily over the years[274]. Therefore, interventions explicitly aimed at interrupting transmission by targeting both parasite and vector will be instrumental in eliminating transmission foci[2]. Transmission-blocking vaccines (TBVs) are one such intervention that seeks to inhibit parasite infectivity in the mosquito. Though TBVs have been deemed 'altruistic' as they do not offer direct protection to the recipient, their ability to offer community-level protection can have substantial public health benefits[10,275]. Successful implementation of a TBV has been seen with Leishmune®, a vaccine against canine visceral leishmaniasis (CVL). CVL is caused by *Leishmania infantum*, an obligate intracellular parasite transmitted via female phlebotomine sand flies[276]. Dogs are the predominant host; however, human infections do occur. Leishmune® is licenced for use, conferring herd immunity to dogs and potentially reducing disease incidence in humans[277,278]. TBVs are also actively being pursued as interventions for other vector-borne diseases such as schistosomiasis[279] and mosquito-borne arboviruses[280].

In 1976, Carter and Chen demonstrated the ability of transmission-blocking immunity to significantly reduce oocyst development in mosquitoes using a *P. gallinaceum*-chicken model of infection[281]. Using purified X-irradiated gametes to vaccinate chickens before parasite challenge, they induced immune responses that inhibited parasite infectivity to mosquitoes. This provided early evidence to support

the development of TBVs for malaria. The existence of naturally acquired transmission-blocking immunity (NA-TBI), further supports TBV development. Surface antigens on gametocytes are targets of naturally acquired antibodies. These naturally acquired, anti-gametocyte antibodies do not confer protection to the host, but when taken up with the blood meal prevent gamete development or fertilisation. Unfortunately, TBV development has lagged behind that of pre-erythrocytic and erythrocytic vaccines, as evidenced by only two candidate antigens tested in phase Ia/b clinical trials to date. In the past decade, however, there has been renewed interest in developing TBVs, and work is ongoing to identify new target antigens. A detailed description of parasite-based and mosquito-based targets follows below.

1.6.3.1. Parasite-based TBV antigen targets

Parasite-based sexual stage antigens can either be pre- or post-fertilisation antigens. Pre-fertilisation antigens are expressed in the human host and hence induce antibody responses after natural infection; however, these antibodies only exhibit function once within the mosquito midgut (reviewed in Stone et al. (2016)[27] and Sauerwein and Bousema (2015)[282]). On the other hand, post-fertilisation antigens are expressed in zygotes and ookinetes within the mosquito hence are not targets of naturally acquired antibodies (reviewed in Sauerwein and Bousema (2015)[231]). The lead TBV candidates are the widely studied pre-fertilisation antigens Pfs230 (epitope-containing protein fragment termed region C), Pfs48/45 and the post-fertilisation antigen Pfs25. Prioritisation of these candidates for development was based on historical observation rather than superior TBA.

Pfs230 is expressed on the surface of male gametocytes and microgametes[283]. Upon gametocyte emergence from the iRBC, the N-terminal portion is cleaved and the C-terminal portion retained on the gamete surface where it is critical to the formation of exflagellation centres required for male gamete maturation[36,284,285]. Pfs48/45 is expressed on both micro- and macrogametes and is thought to be required for gamete fertility and aiding gamete membrane fusion to form zygotes[286–288]. Pfs48/45 is needed to retain Pfs230 on the gamete surface; this interaction is vital for fertilisation[36]. Pfs48/45 is still in pre-clinical development with promising results[289–291], while Pfs230 has entered Phase Ia testing with the release of results on candidate

Pfs230D1M-EPA/Alhydrogel pending (ClinicalTrials.gov Identifier: NCT02334462) and trial of candidate Pfs230D1M-EPA/AS01 (NCT02942277) underway.

Pfs25 is expressed on zygotes and ookinetes and is thought to aid the ookinete in penetration of the midgut[292–294]. Clinical trials of Psf25 have so far not been very encouraging, with reduced immunogenicity and the induction of short-lived antibody being the main challenges. Pfs25 has been evaluated in formulation with different adjuvants in a bid to enhance immunogenicity in humans. A phase Ia trial of Pfs25 with Montanide ISA 51 led to severe reactogenicity causing early termination of the trial[295]. An improved formulation of Pfs25 conjugated to *Pseudomonas aeruginosa* exoprotein A (EPA) adjuvanted in Alhydrogel was well tolerated and immunogenic; however, antibody titres declined rapidly over time[296]. A phase Ib trial in Malian adults induced much lower titres[197]. After four vaccine doses, only 27% of participants had antibody titres with significant blocking activity which again waned rapidly. Novel formulations, i.e. using liposomal adjuvant Glucopyranosyl Lipid Adjuvant (GLA-LSQ) that contains TLR ligands[297] or adenoviral vectors (ChAd63 Pfs25-IMX313 and MVA Pfs25-IMX313)[298] have shown promise in rodent studies and are now undergoing clinical evaluation.

Importantly, NA-TBI has been observed in the absence of antibody responses to Pfs230 and Pfs48/45, and immune recognition of either of the two antigens does not always correlate with TBI[74,205,214,299,300]. This is a clear indication that there are other antigens important for NA-TBI, and hence their identification and characterisation will allow more rational prioritisation of TBV candidates for development. With the elucidation of the molecular interactions essential to various stages of parasite development within the mosquito midgut and the parasite proteins involved, additional antigens have been identified and evaluated for TBA (**Table 1.2**). Moreover, research is underway to identify new candidate antigens. Approaches taken include (1) investigating antigens with a crucial role in sexual stage development, as determined from rodent malaria gene knockout studies[301–303], or (2) proteomic and bioinformatic analyses of parasite genes to identify potential sexual stage antigens[74,304]. Identifying new targets will increase the number of antigens for testing and provide an opportunity for the identification of synergistic antigen combinations that may improve TBV efficacy.

1.6.3.2. Mosquito-based TBV (mTBV) antigen targets

Mosquito receptors that interact with the parasite ligands during sporogonic development also provide attractive targets for TBV development. A significant advantage of mosquito-based TBVs (mTBVs) is the ability to inhibit diverse species of *Plasmodium* by targeting conserved molecules[305,306]. mTBVs can reduce the reproductive capacity of mosquitoes and/or their survival, thus increasing the impact of a TBV by reducing vector populations[305,307]. Early studies demonstrated reduced parasite development after mosquitoes were fed on blood meals containing antibodies raised against midgut homogenate[305,308]. Further work identified mosquito glycans as key mediators of parasite midgut invasion[306].

Additionally, a conserved midgut-specific alanyl aminopeptidase (AnAPN1) was identified, with anti-AnAPN1 antibodies blocking the invasion of both *P. falciparum* and *P. berghei*[309]. Further investigations of ANAPN1 have yielded variable results[232,289]; more studies are warranted to confirm its efficacy. Aside from AnAPN1, the midgut antigen Carboxypeptidase B1 has also been demonstrated to induce transmission-blocking antibodies[307,310]. Additionally, the identification of Saglin, an *Anopheles* salivary gland ligand involved in sporozoite invasion of the mosquito salivary gland provides yet another TBV target for investigation[311,312]. Furthermore, Manning *et al*. (2020) have developed the *Anopheles gambiae* saliva vaccine (AGS-v) that is composed of four *An. gambiae* salivary peptides. A phase I trial of this vaccine has proven safe and well-tolerated, paving the way for further studies aimed at investigating the transmission-blocking potential of mTBVs.

1.6.4. Combination vaccines

A multistage malaria vaccine containing antigens spanning the entire parasite lifecycle may offer the key to achieving long-lasting sterile protection[263]. For instance, the combination of TBV antigens with pre-erythrocytic or blood-stage antigens (or both) in a vaccine would impact transmission and protect against disease while reducing the spread of escape mutants[275]. To support this, Sherrard-Smith *et al*. (2018) showed synergistic action of a PEV and a TBV that resulted in malaria elimination in a murine population assay[313]. Also, the chimeric vaccine GMZ2 plus Pfs48/45, formulated with a TLR-based adjuvant, induced both humoral and cellular responses in mice, and is being progressed for testing in humans[314,315].

Another approach is to use a mixture of recombinant proteins from different parasite stages in a vaccine cocktail. For example, VAMAX-Mix combination vaccines containing AMA1-DiCo with Pfs25 and either CSP, MSP1 or CelTOS produced antibodies specific to each antigen which inhibited the various targeted stages of development[316]. However, though VAMAX-mix combinations showed potent inhibition of transmission and asexual parasite growth, lower efficacy was seen for sporozoite invasion. Whilst this indicated a need to optimise the pre-erythrocytic components of the vaccine, it also highlights the benefits of incorporating multi-stage targets. Failure to achieve complete blockade at one stage can be compensated for as the parasite progresses through the lifecycle by antibodies to subsequent asexual or sexual stages.

However, considerations must be made on the compatibility of antigens and vaccine platforms to avoid reduced efficacy due to immunological interference. Evaluation of the co-administration of RTS,S/AS01B and ME-TRAP demonstrated lower vaccine-induced responses to RTS,S following co-administration[317]. In this study, the ME-TRAP was delivered in viral vectors using a heterologous prime-boost strategy. Therefore, though multi-stage combination vaccines are a promising approach, several hurdles relating to vaccination platform, choice of adjuvant, antigen selection, and identification of clear correlates of protection need to be overcome[263].

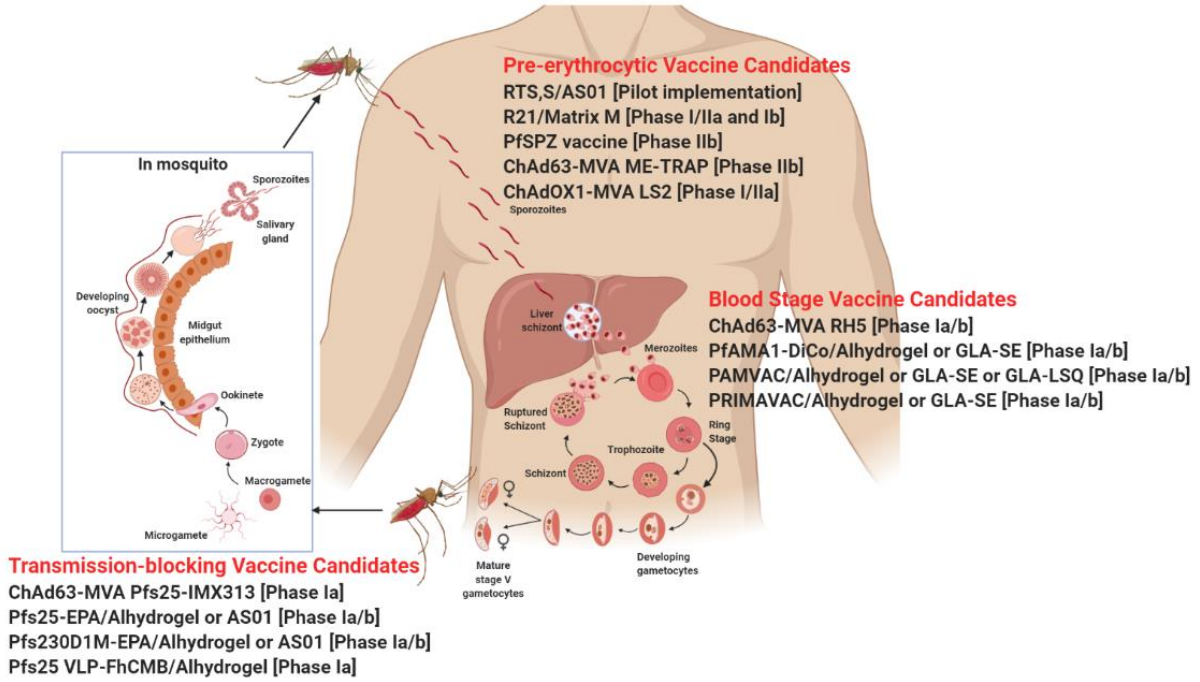


Figure 1.2: Summary of malaria vaccine candidates under clinical evaluation. Summary of some of the vaccine candidates under evaluation, with an indication of the lifecycle stage to which they are targeted. Data summarised from Draper *et al.* (2018)[236] and obtained from the WHO Malaria Vaccine Rainbow Table, and Clinicaltrials.gov.Image created using ©BioRender (https://app.biorender.com/)

Table 1.2: Summary of some well-studied TBV candidates

Antigen	Expression Stage	Role	Advantages	Disadvantages	References
Pfs230	Pre-fertilisation	-Formation of exflagellation centres, interaction with Pfs48/45	-Recognised by immune sera[a] -Antibodies show TBA[b] mainly mediated by antibodies against Region C	-Immune recognition does not always predict transmission blockage -Challenge for full-length protein expression due to large size.	27,36,74,217,283
Pfs48/45	Pre-fertilisation	-Fertilisation of macrogamete, interaction with Pfs230	-Recognised by immune sera -Antibodies show TBA	-Immune recognition does not always predict transmission blockage - Challenge with expression of correctly folded full-length protein	27,74,217,287
Pfs47	Pre-fertilisation	-Non-essential role in female gamete fertility -Mediates evasion of the mosquito immune system	-Recognised by immune sera -Antibodies show TBA	- Currently unknown whether immune recognition correlates with TBA	86,220,318
HAP2	Pre-fertilisation	-Important for male gamete fertility	-Recognised by immune sera -Antibodies show TBA	- Challenge with expression of correctly folded full-length protein - Currently unknown whether recognition correlates with TBA	207,221,319
CCp proteins	Pre-fertilisation	-Important for oocyst sporozoite transmission to salivary gland	-Antibodies show TBA	- TBA not tested in human malaria - Currently unknown whether recognition correlates with TBA	320,321
Pfs25	Post-fertilisation	-Ookinete invasion of midgut by binding to host epithelial receptors.	-Antibodies show TBA -Not subject to immune pressure in human host	-Not expressed in human host therefore no natural boosting of antibodies -Poor immunogen	292,322,323

Antigen	Expression Stage	Role	Advantages	Disadvantages	References
Pfs28	Post-fertilisation	-Ookinete invasion of midgut by binding to host epithelial receptors.	-Antibodies show TBA -Not subject to immune pressure in human host	-Not expressed in human host therefore no natural boosting of antibodies -Poor immunogen	[293,324]
Chitinase (CHT1)	Post-fertilisation	-Ookinete invasion of midgut peritrophic matrix[c]	-Antibodies show TBA	-Limited studies in *P. falciparum* infection model	[325,326]
CTRP	Post-fertilisation	-Ookinete invasion of midgut by binding to host epithelial receptors.	-Antibodies show TBA	-Limited studies in *P. falciparum* infection model	[326,327]
WARP	Post-fertilisation	-Ookinete invasion of midgut by binding to host epithelial receptors.	-Antibodies show TBA	-Limited studies in *P. falciparum* infection model	[326,328]
CelTOS	Post-fertilisation and pre-erythrocytic	-Ookinete and sporozoite traversal of the epithelial lining of the mid-gut and hepatocyte respectively	-Antibodies show TBA -Potential as both a pre-erythrocytic and transmission-blocking vaccine candidate.	-Low to moderate TBA (though fewer ookinetes are able to penetrate midgut some still form oocysts with infective sporozoites)	[329,330]
AnAPN1	Mosquito midgut	-Mosquito midgut ligand mediating parasite invasion.	-Antibodies show TBA against multiple *Plasmodium* species -Highly conserved among *Anopheles* species	-Has been shown to exhibit variable TBA with antibodies raised against N-terminal	[232,289,309]
Carboxypeptidase B1	Mosquito midgut	-Mosquito cofactor (digestive protease) required for parasite development	-Antibodies show TBA against multiple *Plasmodium* species -Highly conserved among *Anopheles* species.	-Targeting a mosquito digestive enzyme may reduce reproductive fitness of the vector therefore selecting for resistance	[307,310]
Saglin	Mosquito midgut	- Involved in sporozoite invasion of the mosquito salivary gland	-Antibodies shown to block salivary gland invasion	-Potentially one of several receptors mediating salivary gland invasion	[311,312,331]

Antigen	Expression Stage	Role	Advantages	Disadvantages	References
				-Not highly conserved among Anopheline species	

TBA- transmission-blocking activity, CCp – Limulus coagulation factor C (LCCL) domain-containing protein, CTRP – circumsporozoite and thrombospondin-related adhesive protein (TRAP)-related protein, WARP – von Willebrand adhesive domain-related protein, AnAPN1 – *Anopheles* alanyl aminopeptidase N1

[a] Immune sera describes sera from individuals living in malaria-endemic areas

[b] Antibodies against Pfs230 work in a complement-dependent manner[204,206].

[c] The peritrophic matrix is a chitinous layer surrounding the midgut formed after the mosquito bloodmeal[332]

1.7. Antigen discovery for vaccine development

The majority of the malaria vaccine candidate antigens under investigation in clinical trials were identified prior to the publishing of the *P. falciparum* genome. Candidates were thus identified by screening genomic or cDNA expression libraries for immunoreactive proteins, or by the characterisation of proteins identified by immunoprecipitation analysis. As a result, antigen discovery was slow, and few candidates were identified. The publishing of *P. falciparum*'s genome in 2002[333] and the characterisation of stage-specific proteomes[334] heralded the beginning of the genomic era of vaccine candidate discovery. Using 'reverse vaccinology', the identification of potential antigens begins *in silico* using computer software to predict antigenic targets based on genomic data, these targets are then evaluated for a role in protective immunity[335]. Successful use of this approach was first demonstrated with Group B *Meningococcal* (MenB) vaccine candidate discovery. After over forty years of stalled progress, sequencing of the MenB genome provided a means to identify over 600 potentially surface localised proteins[335–337]. Following cloning, protein expression, antibody production and functional assays, 25 new candidates were identified in 18 months culminating in the development of a licenced multi-component protein-based vaccine, 4CMenB[335,338]. Significant technological breakthroughs have since followed, including: (1) the ability to clone human B cells to produce monoclonal antibodies (mAbs) or antibody-binding fragments to interrogate the protective immune response, (2) structural biology to map conformational epitopes, and (3) the ability to design immunogens based on structural and immunological data that target specific epitopes to elicit a defined immune response (reviewed in Rappuoli *et al.* (2016)[339]). With the aid of these advances, several reverse vaccinology approaches have now been developed and used to discover novel antigens for evaluation, and they are discussed here below.

1.7.1. Pre-genomic era

1.7.1.1. Screening of genomic or cDNA libraries

In order to develop genetic libraries, genomic DNA or cDNA (prepared from the nucleotide protein precursor messenger RNA) are prepared by enzymatic digestion and subsequently cloned into expression vectors[340]. Recombinant protein corresponding to individual clones can then be synthesised in bacteria or phages and

after probed with hyperimmune sera to identify immunoreactive proteins and their corresponding genes. Recombinant CSP, the protein on which the RTS,S vaccine is based, was first produced in this way using mRNA from *P. knowlesi* infected *Anopheles dirus* thoraxes[341]. Since then other candidate antigens have been identified in this way, e.g. schizont egress antigen[185] and liver stage antigens 1[342] and 3[343]. One of the advantages of this approach is that it allows an unbiased screen of the proteome potentially increasing the number of immunoreactive proteins identified. On the other hand, this approach is subject to the sera used and the expression platform chosen[340].

1.7.1.2. Immunoprecipitation

Prior to the use of recombinant DNA technology to produce antigens for characterisation, stage-specific parasite proteins were obtained from lysates prepared from cultured parasites. Monoclonal antibodies (mAbs) were then produced using spleen cells derived from mice vaccinated with purified stage-specific parasites using hybridoma technology. The mAbs would then be used in functional assays to assess inhibitory activity, and to immunoprecipitate radioisotope-labelled parasite lysate run on SDS-PAGE. In this way, the first TBV candidates Pfs25, Pfs230, and Pfs48/45 were identified, with their names denoting their molecular weight upon separation by SDS-PAGE[208,344,345], as was the merozoite vaccine candidate MSP3[346]. The availability of proteomic data in the post-genomic era allows this approach to be taken further by allowing the unambiguous identification of antigens of interest for further evaluation.

1.7.2. Post-genomic era

1.7.2.1. Functional comparative genomics

Functional comparative genomics involves comparing gene families between species to identify homologues that may perform similar functions. Prior to the availability of the complete genomes of various *Plasmodium* species, functional comparison of gene families had already proved its usefulness. Members of the *P. falciparum* RH family that encompasses reticulocyte binding proteins (RBPs) involved in merozoite invasion of RBCs, were identified by identifying homologues of *P. yoelii* and *P. vivax* RBP families[347–349]. These were identified by amplifying and sequencing single genes for comparison. A member of the *P. falciparum* RH family, RH5, is now a promising vaccine candidate antigen. Furthermore, by exploiting publicly available genome

data, Frech and Chen (2011) performed a comparative analysis of *P. vivax* and *P. falciparum* genomes using bioinformatic tools[350]. The authors identified a subset of genes present only in *P. falciparum* linked to virulence, with 15 of these uncharacterised, hence potentially offering new vaccine candidate leads.

1.7.2.2. Population genetic analysis (PGA)

With population genetic analysis, polymorphic parasite genes that are under balancing selection, due to factors such as immune pressure, are identified by comparing genomes within or between populations[340,351]. This is supported by the assertion that highly polymorphic genes such as AMA1 and MSP1 are targets of NAI and have been associated with protection against clinical malaria in seroepidemiological studies[182–184]. Ochola *et al*. (2010) used transcript data from 13 isolates available on the *Plasmodium* database, PlasmoDB, to identify polymorphic genes from the blood stage of the parasite[352]. Using a minimum of five single nucleotide polymorphisms (SNPs) per kilobase as the cut-off, they prioritised six highly polymorphic loci from the schizont/merozoite stage. The authors further analysed polymorphisms at each of the six loci in 90 Kenyan field isolates leading to the identification of three antigens ($SURFIN_{4.2}$, MSPDBL2, and MSPDBL1) with evidence of strong balancing selection. Antibodies against the MSPDBLs have been shown to inhibit merozoite invasion[353] while $SURFIN_{4.2}$ is thought to have a role in mediating rosetting[354], making these antigens potential vaccine candidates.

Similarly, Amambua-Ngwa *et al*. (2010) used high-throughput next-generation sequencing to analyse parasites isolated from 65 clinical isolates from an endemic region in Ghana[355]. In this way, they identified over 300 genes with evidence of balancing selection. Identified targets included known immunogenic proteins, such as AMA1 and MSP3, the previously identified $SURFIN_{4.2}$, MSPDBL2, and MSPDBL1, as well as a host of novel antigens. PGA, therefore, can lead to the simultaneous identification of several antigens for evaluation; however, downstream analysis will be required to prioritise candidates for functional studies. Additionally, polymorphic targets have not always fared well as vaccine candidates, i.e., AMA1 and MSP1, which may limit the utility of this approach.

1.7.2.3. Transcriptomics

The transcriptomic approach is based on the premise that the specific parasite life cycle stages or phenotypes will upregulate a specific set of genes corresponding to potential vaccine targets[356]. Traditional methods relied on DNA microarrays where chips containing DNA are probed with fluorescently labelled cDNA prepared from mRNA derived from the sample of interest. However, the advent of RNA sequencing now allows for more accurate and sensitive quantification of transcripts and the identification of polymorphic targets as well as splice variants (reviewed in Tuju *et al*. (2017)[340]). Differential transcriptomics enabled the identification of the PAM vaccine candidate *var2csa*. A comparison of parasites binding to CSA, the ligand implicated in the binding of iRBCs to the placenta, with non-CSA binding parasites revealed that *var2csa* was upregulated in CSA-binding parasites[46].

Further work went on to confirm its role in PAM. This approach has also been used to investigate the *var* transcriptome of parasites isolated from children with severe malaria in comparison to those with mild malaria or asymptomatic children in a bid to identify genes associated with pathogenesis. While distinct subsets appear to be upregulated in severe malaria[56,357,358], translating this to a candidate antigen is hampered by the highly polymorphic nature of this protein family. Furthermore, unlike with PAM, where an apparent phenotype exists, the pathogenesis of severe malaria is complex making it challenging to link certain parasite traits with disease manifestation definitively.

1.7.2.4. Proteomics

Proteomics is the identification and quantification of the entire complement of proteins produced in an organelle, organ, or organism[340]. The proteome is not constant but varies temporally, by cell type, and in response to environmental stimuli. Recent proteomic studies have identified several proteins expressed during the sexual stages within the human host[359–361] and the anopheline vector[362]. Silvestrini *et al*. (2010) through the comparative analysis of the proteomes of trophozoites, early gametocytes, and late gametocytes identified proteins highly enriched in each of these stages, with 637 exclusively expressed in stage V gametocytes[363]. Moreover, computational analyses of the proteomes of gametocytes[360], microgametes, and ookinetes[362,364] have provided datasets that can be mined to identify possible surface-localized proteins.

An alternative to whole-organelle or whole-organism proteomics is membrane proteomics where proteins on the surface of parasite stages are extracted, separated, and identified by mass spectrometric techniques. This was the method used to analyse the surface of the ookinete, revealing 50 surface-localised proteins for further analysis[362]. Proteomics is a powerful tool allowing the identification of several antigens, thereby necessitating the use of rational criteria for candidate antigen selection.

1.7.2.5. Immunomics

Immunomics combines genomic, transcriptomic, proteomic, and immunologic methods, using biological samples from malaria-exposed humans or animals to identify protective antigens or epitopes[365,366]. Furthermore, immunomics considers the entire immune response; antibody titre, cytokine production, T cell population induced among others[366]. Different approaches can be taken to identify vaccine candidates. For instance, proteome-wide screening assays can be used to interrogate humoral[74,156,227,367,368] or cellular[369] responses in experimentally infected or naturally exposed individuals. Antigen selection for immunoscreening can be unbiased, where no filtering criteria are used to select candidates, or biased towards selecting immunogenic proteins.

Surface-exposed proteins can be identified based on features predictive of secretion or surface localisation. Such features include the presence of signal peptides, transmembrane (TM) domains, and glycosylphosphatidylinositol (GPI) anchors[74,304]. Stone *et al.* (2018) identified novel potential gametocyte TBV candidate antigens using a protein microarray platform[74]. Sera from 648 malaria-exposed individuals were tested against a panel of 315 proteins enriched for gametocyte-specific proteins, and commonly recognised antigens among individuals with high TBA identified. The authors were thus able to highlight 13 novel antigens with a potential role in TBI. Such studies into novel sexual stage antigens, therefore, have the potential to reveal new targets for TBV development. However, challenges with protein expression as well as the biased approach to antigen selection may leave out promising candidates. Results of such studies should thus serve to rule in rather than rule out candidate antigens.

In an unbiased approach, two-dimensional gel electrophoresis is used to separate protein fractions derived from parasite lysate which are then subsequently transferred onto membranes where they can be probed using sera from malaria-exposed vs malaria naïve individuals. This approach was used by Fontaine *et al*. (2010) to evaluate iRBC antigenic targets[370]. By extracting from a non-serum-probed gel the proteins corresponding to differentially recognised antigens and subjecting those proteins to mass spectrometry, they could identify promising proteins for further evaluation. Technical challenges relating to the use of denaturing conditions that destroy conformational epitopes, as well as incomplete protein transfer, may decrease detection power. Nevertheless, immunomics is undoubtedly a promising technique for antigen discovery through its integration of 'omic' and immunologic data and the application of high throughput technologies for screening.

1.7.2.6. Antibody-guided candidate discovery

Antigen identification using the strategies above does not guarantee that the identified immunogenic protein is functional in an *in vitro* or *in vivo* setting[340]. In order to circumvent this, protective epitopes can be identified using a variety of epitope prediction software, and the corresponding antigens elucidated for analysis. Doolan *et al*. (2003) used this approach to identify potential T cell epitopes from the sporozoite genome and then synthesised peptides corresponding to the antigens containing these epitopes for protein microarrays[369]. The authors then probed the microarray with sera from individuals challenged with radiation attenuated sporozoites and sera from naïve individuals. Sixteen previously uncharacterised antigens preferentially recognised by the challenged individuals were thus identified as potential vaccine candidates.

Aside from using epitopes, broadly reactive monoclonal antibodies prepared from the sera of malaria-immune individuals can be used to design mimotopes for evaluation as immunogens[340]. This borrows from the field of HIV research where broadly neutralizing antibodies have been well characterised and successfully produced, with the challenge now being how to formulate immunogens to stimulate the production of such antibodies (reviewed in Rappuoli *et al*. (2016)[339]). Broadly reactive mAbs capable of agglutinating diverse parasite isolates were identified by Tan *et al*. (2016) from the B cells of malaria-exposed donors[371] lending credibility to the use of this

approach for malaria. Limitations are that such broadly-reactive antibodies form a small percentage of the immune repertoire, and individuals harbouring them tend to be rare[340]. This necessitates the screening of large panels of sera which is technically demanding. Nevertheless, designing immunogens that recapitulate the epitopes targeted by such antibodies or that stimulate B cell precursors of these broadly neutralising antibodies presents a new approach to vaccine candidate discovery.

1.8. Assessment of TBV efficacy

Evaluation of malaria vaccine candidate antigens can be achieved using laboratory assays that test for surrogate markers of protection, CHMI studies in malaria-naïve and malaria-exposed individuals as well as field trials in endemic populations. Laboratory assays to test the ability of candidate antigens to induce immune responses functional against cultured parasites typically form the first level of evaluation. An example is the growth inhibition assay (GIA) used to assess blood-stage antigens. While the GIA measures an important component of the functional immune response, it often fails as a correlate of protection in seroepidemiological studies owing to a lack of reproducibility and dependence on lab-adapted parasite strains[372]. Vaccine-induced immunological correlates of protection that are robust, reproducible, easy to measure, and well correlated with vaccine efficacy would greatly facilitate vaccine development.

From laboratory assays, evaluation moves to animal models and CHMI studies where additional parameters such as achievement of sterilising protection, delays to patency, reduction in parasite burden or parasite multiplication rate[176,179,239,373] (PEVs and BSVs) or reduced infectiousness to mosquitoes (TBVs)[374,375] can be assessed. After this, efficacious candidates can be progressed to field trials in endemic settings where the impact in reducing disease burden and transmission can be evaluated[248,257,376]. For TBVs, efficacy is assessed as the ability to reduce or inhibit parasite development within the mosquito, mainly using mosquito feeding assays. This is typically achieved by feeding gametocyte-infected blood or RBCs (for cultured parasites) to mosquitoes in the presence or absence of sera containing inhibitory antibodies and enumerating the number of oocysts that eventually develop within the midgut. Another measure of success is in the reduction in the numbers of mosquitoes infected in comparison to a negative control. Mosquito feeding assays have the advantage of interrogating

antibodies that act on various stages of sexual development in the midgut, from mature gametocyte to oocyst, making them valuable tools[377]. Commonly used TB assays to evaluate TBVs are described below.

1.8.1. Field-based methods

Field-based methods for TBV assessment use mosquito feeding assays where gametocyte-infected blood from donors is the parasite source. The advantages of field evaluation are the assessment of blockade at naturally circulating parasite densities and the use of locally circulating parasite strains[98,378,379]. Moreover, autologous plasma can be used as the antibody source to assess natural or vaccine-induced TBI, providing more realistic estimates of efficacy. Alternatively, heterologous plasma from different donors or purified immunoglobulins derived from vaccinated animals can replace the autologous plasma in what is referred to as serum replacement. Serum replacement is desirable if the presence of drugs or serum components may influence TBA readout[379]. Feeding can be done directly from the skin of test subjects (direct feeding assay, DFA) or venous blood can be fed to mosquitoes through a membrane (direct membrane feeding assay, DMFA). Transmission efficiency is reportedly higher in the DFA as compared to the DMFA[375,380,381]. The reason for this is still unclear. One hypothesis put forward to explain this difference is that the sequestration of parasites in the microcapillaries may facilitate uptake by skin feeding; however, there is no direct evidence to support this. Early studies in the Congo compared gametocyte densities between skin snips and peripheral blood, reporting higher densities in the skin snips[382,383]. However, recent investigations using molecular parasite detection techniques and paired skin and venous blood samples from the same donor have not shown evidence of gametocyte sequestration in the skin[384,385]. Another hypothesis cites technical challenges with maintaining blood at 37°C during DMFA that may lead to premature activation of gametocytes, thus lowering their infectivity[378,381] or damage induced by venepuncture[381]. Further investigation into the enhanced infectivity of skin feeding is warranted.

Though DFAs result in better infectivity, ethical considerations preclude skin feeding on young children hence DMFAs are preferred where all age groups are under investigation. Other advantages of DMFAs over skin feeding are that (1) more mosquitoes can be included thereby increasing statistical power, (2) gametocyte

densities can be quantified in the bloodmeal, (3) bias in an individual's attractiveness to mosquitoes is eliminated, and (4) experimental modifications, e.g. serum replacement, are easier to achieve[378,379]. Though DFAs and DMFAs are challenging to standardise within and between labs owing to host, vector and procedural effects, measures can be taken to mitigate against this[378]. Such measures include developing gold standard methodological practices that could serve as a reference. Additionally, standardising donor selection criteria and collecting relevant information on host-related confounders may mitigate against host effects. Using locally reared mosquito colonies would also guard against any parasite-vector incompatibilities that may reduce infectiousness. The utility of DFAs and DMFAs for evaluating TBV candidates in field settings is undeniable. Therefore, the development of standardised protocols will allow for better reproducibility and the broader application of these tools.

1.8.2. Laboratory methods

1.8.2.1. Gamete formation inhibition assays

Transmission-blocking vaccines and drugs can inhibit parasite development by blocking the formation of mature male or female gametes. Differentiation into male and female gametes in the mosquito midgut requires egress from the host RBC signalled in response to environmental stimuli[26] (described in detail in **1.1.2.3.** above). Antibodies to vaccine candidate antigens that act by inhibiting male gametogenesis or fertilisation can be assessed by their ability to inhibit exflagellation[321,377,386,387]. The exflagellation assay has been used widely to assay transmission-blocking drugs[377,388,389]. Inhibition of female gamete formation has not been actively studied; however, an assay that assesses rounding of macrogametes in the presence of these drugs has been developed. The assay termed the *Plasmodium falciparum* dual gamete formation assay (*Pf*DGFA) measures inhibition of both exflagellation and macrogamete formation in the same well, allowing delineation of sex-specific inhibition[377]. The limited biological range of the *Pf*DGFA makes it unsuitable for assessing antibodies that act on later developmental stages. However, the assay can provide vital information on the possible function of the antibody target that can be refined by further investigation.

1.8.2.2. Standard membrane feeding assays (SMFAs)

Unlike the DFA and DMFA, the SMFA measures infectivity using cultured parasites as a source of gametocytes. The SMFA is currently the 'gold standard' TBV assay, and it has undergone standardisation to allow for better reproducibility[390–392]. While offering good reproducibility to a certain extent, the SMFA currently does not support the assessment of activity against parasite strains found in the field[378]. Moreover, the gametocyte densities used in bloodmeals may not reflect densities found in the blood of gametocyte carriers. Therefore, activity in the SMFA may not accurately depict expected efficacy in the field, unless considerations are taken to examine multiple parameters such as varied parasite densities and strains. This would be a technically demanding exercise, exacerbating the already labour-intensive MFAs that rely on the dissection of several mosquitoes to achieve precise estimates of efficacy. To circumvent this, a scalable SMFA that uses luminescent parasite expressing luciferase throughout its lifecycle has been developed and allows the assessment of TBA in pools of homogenised mosquitoes[393,394]. Estimates of the intensity of infection are averaged over the pool; hence the calculation of individual-level estimates, as well as estimates of reductions in the prevalence of infected mosquitoes, is not possible. Nonetheless, a high-throughput, semi-automated SMFA increases the testing capacity and efficiency, which would be required for large-scale population-level trials[394].

1.8.2.3. *In vitro* ookinete conversion assay (IVOA)

Production of *P. berghei* ookinetes can be assessed in the presence of transmission-blocking antibodies, and the impact of these antibodies on ookinete development used as a measure of TBA[395,396]. Using a fluorophore-conjugated monoclonal antibody against Pbs21[397], that accumulates on the surface of macrogametocytes and ookinetes[377,395], the rate of ookinete conversion can be estimated from the original number of macrogametocytes. Furthermore, automation of the *in vitro* ookinete conversion assay (IVOA) to increase throughput is possible using the *P. berghei* fluorescent parasite strain PbCTRPp.GFP[398]. PbCTRPp.GFP expresses green fluorescent protein (GFP) under the circumsporozoite- and TRAP-related protein (CTRP) promoter, with maximal expression observed in the mature ookinete stage. Delves *et al*. (2012) have developed a 384-well plate-based assay to screen drugs with TBA, where inhibition of ookinete conversion is one of the parameters measured[396]. Transmission-blocking vaccines can also be screened using this platform. Application

of the IVOA to TBV screening is currently limited to the evaluation of *P. berghei* candidate antigens due to the inability to produce P. *falciparum* ookinetes in culture effectively. *In vitro*, ookinete conversion rates for *P. falciparum* can range between 0.45 to 16% as opposed to 50% estimated for *in vivo* conversion (reviewed in[399]). Improvements in culture protocols, however, will allow the use of the IVOA in *P. falciparum* as well[399,400].

1.8.2.4. Murine population assays

The murine population assay (MPA) is a multigenerational transmission-based study involving serial passages of *P. berghei* in *An. stephensi* that is used to assess the efficacy of transmission-blocking vaccines or drugs over multiple transmission cycles[313,401]. The MPA provides a model system to study the 'real-world' impact of TB interventions on malaria elimination using a laboratory parasite strain. Blagborough *et al*. (2013) used the MPA to demonstrate that a threshold of 80% reduction in oocyst intensity for TBV development[5] may be too stringent[401]. They showed that an intervention with an estimated 57% efficacy was able to eliminate *Plasmodium* in both host and vector populations at a simulated low transmission intensity setting over successive transmission cycles.

Additionally, using the MPA Sherrard-Smith *et al*. (2018) demonstrated synergy between a partially effective PEV and a TBV that eliminated malaria from mouse and mosquito populations. Transmission was eliminated when the PEV and TBV were combined, using an anti-CSP mAb at 47.2% efficacy and anti-Pfs25 mAb-4B7 at 50%, 65% and 85% efficacy, at all TBV efficacies tested. As the efficacy of both vaccines is parasite dependent, the authors attributed the synergistic effect to a reduced parasite density in infected mosquitoes accorded by the TBV, which increased the efficacy of the PEV. It remains unclear how well the MPA approximates field settings where additional factors such as NAI, drug treatment, different vector susceptibility, and antigen polymorphisms exist[313]. However, the MPA can be adapted to investigate these heterogeneities, and thus provide valuable information that can feed into the design of future field trials for TBVs.

1.8.3. Controlled Human Malaria Infection (CHMI)

The Controlled Human Malaria Infection (CHMI) model has found application in the testing of drugs and vaccines and as a tool for studying the interplay between immune

responses and parasite dynamics during infection. Research is now ongoing to develop CHMI transmission models to provide a link between laboratory assays and field evaluation. Field evaluation of TBVs will likely involve randomised trials in endemic populations to evaluate reductions in infectiousness and transmission, and cluster randomised trials to assess reductions in malaria incidence[375]. CHMI transmission models may, therefore, offer a rapid and cost-effective means to prioritising TBV candidates for field trials. Parasite challenge can be achieved using infectious mosquito bites (MB), sporozoite inoculation, or via the direct inoculation of blood stage parasites (IBSM). IBSM has the advantage of allowing all participants to develop gametocytaemia at roughly the same time simplifying study design[375,402].

In contrast to CHMI used to test PEVs or BSVs, blood stage infection needs to progress for gametocytes to develop. Therefore, sub curative doses of slow-acting asexual-specific drugs are given to maintain low levels of asexual parasitaemia. Dosing regimens using piperaquine and SP have shown promise in allowing the development of mature gametocytes in a CHMI setting[403]. Mosquito feeding assays are then used to assess vaccine or drug efficacy. A challenge faced by CHMI-transmission models is that induced gametocyte densities may be too low to infect mosquitoes for downstream analysis[375,404]. Optimisation of infection route and dose of inoculum[402] as well as gametocyte enrichment of participant derived blood are being explored[404] to circumvent this. Though optimisation is required before CHMI transmission models can be employed for routine evaluation of TBV candidates, they offer a promising model to facilitate TBV development.

1.8.4. Assessment of efficacy

The transmission-blocking activity observed with different TBV candidates can be expressed as either reduction in the number of infected mosquitoes (oocyst prevalence) or reduction in oocyst burden in the midgut (oocyst intensity). Some researchers describe reductions in oocyst prevalence as transmission-blocking activity and reductions in oocyst intensity as transmission reducing activity (TRA)[405,406]. Others refer to blockade of either oocyst prevalence or intensity as TBA[207,407], others as TRA[74,408]. There is no consensus on what term better represents TBV efficacy, as well as no consensus on whether reductions in intensity or prevalence provide a better indication of impact in the field. The recommendation is, therefore, that efficacy

should be reported as reductions in prevalence and intensity to aid interpretation[391]. For this study, I chose to use TBA as an umbrella term; however, I report both reductions in intensity and prevalence in keeping with reporting recommendations.

Estimates of TBV efficacy are also confounded by the overdispersed nature of oocyst burden[391,409], the level of infection achieved in the control group used as a comparator[391,406,410], as well as a host of experimental variables. Therefore, to increase precision and accuracy, the following measures have been proposed: (1) increasing the number of mosquitoes dissected to increase power to detect differences between controls and vaccine/drug groups[409], (2) carrying out multiple feeds using variable experimental conditions such as different sources of infected blood and varied parasite exposures[391,410], and (3) the use of mixed models for analysis to account for the effect of such experimental variables on the observed infection levels, and provide more robust estimates[391]. To further enhance comparability, where possible, candidate antigens should be tested in the same feed to minimise batch inter- and intra-assay variability[411]. An understanding of the shortcomings of TBV estimates has enabled the development of experimental and reporting criteria that, coupled with a better understanding of transmission biology, will allow better estimation of efficacy.

1.9. Aims and overview

Through this work, I thus aimed to characterise sexual stage antigen targets of transmission-blocking immunity. First, I sought to better understand NA-TBI by assessing what has been determined from previous studies of NAI to the lead TBV candidates. Additionally, I also investigated the determinants of gametocyte carriage at a population level, as this impacts NA-TBI. I then identified a set of mostly uncharacterised sexual stage antigens and evaluated both naturally acquired immune responses and vaccine-induced responses against them. I did this to improve our understanding of NAI that can feed into the design and evaluation of TBV candidates, and also identify novel TBV candidates potentially.

1.9.1. Aims

1. To investigate the dynamics of NA-TBI in African populations exposed to *falciparum*-malaria.
2. To describe the prevalence and distribution of gametocytaemia among children participating in a longitudinal cohort in Coastal Kenya.

3. To identify sexual stage antigens with potential as TBV candidates and:
    a. Determine the seroprevalence and dynamics of gametocyte-specific antibodies in naturally exposed individuals.
    b. Evaluate the functional ability of antibodies generated against the candidate antigens using *in-vitro* and *ex-vivo* assays.

1.9.2. Chapter overview

In **Chapter Two**, I present the results of a systematic review and meta-analysis of naturally acquired immune responses to Pfs230 and Pfs48/45, limited to malaria-endemic African populations. In this chapter, I evaluate the seroprevalence to Pfs230 and Pfs48/45 antigens, as well as population-level factors that influence the acquisition of NAI to both antigens.

In **Chapter Three**, I carry out an epidemiological analysis of the factors influencing gametocyte carriage using a longitudinally monitored cohort based at the Kenyan coast. Here I use data collected over 19 years and across different transmission settings.

Chapter Four describes the identification of gametocyte, gamete, and ookinete antigens using *in silico* analyses of proteomic datasets. The chapter also describes the evaluation of different expression platforms for the production of recombinant proteins corresponding to these antigens.

In **Chapter Five**, I use information gleaned from **Chapter Two** and **Chapter Three** to conduct immunological profiling of the gametocyte-specific antigens identified in **Chapter Four**. I use sera from three different cohorts to (1) analyse seroprevalence to the gametocyte antigens, (2) describe the dynamics of NAI to the antigens in relation to markers of parasite exposure and risk factors for gametocyte carriage, and (3) assess the relationship between responses to the antigens and onward infectiousness to mosquitoes.

Chapter Six describes the functional evaluation of vaccine-induced antibodies to the sexual stage antigens identified in **Chapter Four**. Antibody titre and TBA were evaluated using *in vitro* and *ex vivo* assays in a *P. falciparum* model of infection for the gametocyte antigens and a *P. berghei* model of infection for the gamete and ookinete antigens.

Lastly, in **Chapter Seven,** I summarise the findings from chapters two through seven, assess potential implications of the findings, and provide recommendations for further studies.

Chapter 2

Immune Responses to Gametocyte Antigens in a Malaria-Endemic Population - the African falciparum Context: A Systematic Review and Meta-analysis

2.1 Introduction

Several antigens expressed at different developmental stages of the parasites sexual lifecycle have been previously identified and characterised as transmission-blocking vaccine candidates (refer to **Chapter 1**, section **1.6.3.1.**). In addition to functional characterisation, there has also been interest in understanding naturally acquired immunity (NAI) to pre-fertilisation sexual stage antigens. Various studies across multiple malaria-endemic regions have revealed that antibodies to Pfs230 and Pfs48/45 are detectable in the sera of malaria exposed individuals[74,200–203] and that these antibodies can mediate transmission-blocking activity (TBA)[74,200]. Recent work has also shown immune recognition of the pre-fertilisation gametocyte proteins Pfs47[220] and HAP2[221] indicating that antigens other than Pfs230 and Pfs48/45 are targets of NAI to sexual stage antigens.

A better understanding of NAI to the sexual stages can play a role in identifying and prioritising key antigens for TBV design. Also, such studies can provide insight into host factors to consider when assessing the efficacy of TBVs in various field settings. Moreover, sexual stage immune responses can potentially impact the structure and dynamics of the human infectious reservoir[200] allowing us to identify populations requiring targeted interventions. Most studies have relied on indicators of parasite exposure such as host age, parasite prevalence, malaria transmission setting, and seasonality to define the dynamics of sexual stage immunity. Currently, there is limited consensus on how these factors affect the acquisition of antibodies to sexual stage antigens, and their subsequent impact on vaccine efficacy.

Discrepancies exist, for instance, in the observed association between age and seroprevalence to the sexual stage antigens. The relationship between age and seroprevalence to asexual stage antigens is well described, and it has been shown that immune responses increase with age[412–414]. For sexual stage antigens, some studies have shown no age-dependent increase in responses[217,218] while others have shown an

increase in seroprevalence with age[203,227,228]. Additionally, there are conflicting reports on the association between transmission intensity and anti-gametocyte immunity. From their study in Tanzania, Bousema *et al.* (2007) found lower antibody responses in a high transmission setting compared to a low transmission setting[218]. On the other hand, Amoah *et al.* (2018) found higher seroprevalence in the high transmission setting studied[415]. These differential associations indicate that interaction between the various factors associated with parasite exposure impacts the reported seroprevalence and that these relationships require further exploration.

Furthermore, studies aimed at describing the seroprevalence to sexual stage antigens employ varied study designs (cross-sectional, longitudinal, or rolling recruitment at healthcare facilities) and sampling protocols (for instance studies limited to children or adults only). For these reasons, methodological factors may also affect the reported seroprevalence. A combined analysis of the studies that have analysed naturally acquired immune responses to gametocytes, considering both biological, environmental, and methodological factors is therefore required. This would bring us closer to understanding how prevalent sexual stage immune responses are in the population and what factors are essential in its acquisition.

2.2 Rationale

Owing to their early identification and extensive characterisation over the years, the majority of studies into NAI to sexual stages have examined responses to the six-cysteine-rich protein family members: Pfs230 and Pfs48/45. For this reason, I chose to focus on studies that reported the seroprevalence to either of these antigens. At the time of publication, this was the first attempt to pool data from studies of NAI to Pfs230 and Pfs48/45 to better understand seroprevalence and the factors that influence antibody responses to these antigens. Though Pfs230 and Pfs48/45 are the leading pre-fertilisation TBV candidates, work is ongoing to identify new candidate antigens. There is also evidence of other antigens important for the acquisition of NA-TBI[74,218]. Increased interest in identifying novel targets of NA-TBI will require the identification and validation of essential criteria to prioritise candidate antigens for clinical testing. In addition to generating a better understanding of the factors that influence the development and dynamics of sexual stage immunity, I also aimed to gain insights that would guide the characterisation of novel TBV targets.

2.3 Objectives

The main objective of this systematic review and meta-analysis was to investigate the dynamics of NA-TBI in African populations exposed to *falciparum*-malaria.

2.3.1 Specific objectives

a). Describe seroprevalence to the widely studied gametocyte antigens Pfs230 and Pfs48/45.

b). Identify factors, at a population level, that are associated with the acquisition of naturally acquired anti-gametocyte immunity.

2.4 Methods

I carried out a systematic review of studies in African populations exposed to *Plasmodium falciparum* malaria that assessed and reported the seroprevalence to Pfs230 and Pfs48/45. I used criteria provided by the Meta-analysis Of Observational Studies in Epidemiology (MOOSE) guidelines and the PRISMA (Preferred Reported Items for Systematic Reviews and Meta-Analyses) guidelines to conduct and report the analyses[416,417]. The study protocol is registered on PROSPERO (number CRD42019126701).

2.4.1 Study design

Due to the lack of studies assessing naturally acquired immune responses to gametocyte antigens, I aimed to be as inclusive as possible to capture the majority of studies. For this reason, cross-sectional and longitudinal studies spanning the malaria transmission season were included—this introduced heterogeneity which I mitigated against using meta-regression analyses. As health facility-based studies recruited participants with acute malaria infection, they were not included in the analyses. Concurrent malaria may influence seroprevalence estimates, thereby confounding the results and limiting their generalisability.

2.4.2 Study participants

The study population consisted of individuals living in malaria-endemic areas in Africa exposed to *P. falciparum* infection. Studies that recruited both children and adults are included in the analysis; however, studies in pregnant women were excluded from the analysis. Pregnant women, while typically older than children, are

more susceptible to malaria owing to the possibility of pregnancy-induced immune suppression; thus, their inclusion could potentially confound the analysis. The outcome investigated was the presence of antibodies to Pfs230 and/or Pfs48/45.

2.4.3 Search strategy

The keywords: (pfs230 OR pfs48 OR pfs45) AND (antibodies OR immunity OR response) AND (*Plasmodium* OR *falciparum* OR malaria) were used to search various databases. Additionally, reference lists of identified articles were also searched to identify relevant articles. The databases searched were MEDLINE/PubMed, SCOPUS, Web of Science, African Index Medicus, Embase and African Journals Online. The search was carried out between the 1st of February and the 31st of March 2019. For cases where it was not possible to extract data on seroprevalence directly from the article, I contacted the authors of the articles to request the data. Alternatively, where raw data were readily available in public repositories, I used these data in the analysis.

2.4.4 Study selection

Articles were included in the study if they were: (1) studies conducted in African populations, and (2) studies reporting antibody responses to either Pfs230 or Pfs48/45 or both antigens. I considered studies from all years and written in all languages for review. Articles were excluded if: (1) they only reported antibody responses to non-*falciparum* antigens, (2) they were a vaccine, drug or any other interventional trial, (3) they did not use a quantitative assay to measure immune responses, and (4) they sampled fewer than 30 participants (this included studies where both children and adults were recruited, but there were fewer than 30 participants in each category). In cases where there were two studies carried out in the same cohort, I considered the study where seroprevalence estimates were analysed in relation to a larger number of the variables investigated in the analyses.

2.4.5 Data extraction

Data on seroprevalence to Pfs230 and Pfs48/45 were extracted from relevant articles using a standardised data extraction form. The form was designed to capture information on the following variables: study design employed, country and location (village, district or other administrative unit) of where the study was carried out, malaria transmission intensity of the study site, the season during which the

participants were recruited and immune responses measured, asexual and sexual parasite prevalence in the study population, study population investigated (children or adults), age categories investigated, type of immunoassay used to detect immune responses, antigen coating concentration, serum dilution, source of antigen for the immunoassay, type of negative controls used, and method used to assess seropositivity.

2.4.6 Data analysis

Between-study heterogeneity was assessed using Cochran's Q, I^2 and H statistics. I^2 cut-offs of <30%, 30-75%, and >75% were used to defined low, moderate, and high estimates of heterogeneity, respectively. Moderator analysis using sub-groups and meta-regression were used to explore potential sources of heterogeneity. The strength of the associations observed in the meta-regression was assessed using conservative *p* values calculated using the 'Knapp-Hartung' method. Additionally, to correct for multiple comparisons, adjusted *p* values were calculated using the Benjamini and Hochberg correction[418]. I calculated the change in heterogeneity score after carrying out each univariable analysis using the formula:

[(overall heterogeneity – residual heterogeneity/overall heterogeneity) * 100]

This was done to identify variables associated with higher levels of heterogeneity.

In the meta-regression, the following variables were explored: (1) age group – classified broadly as children (from 0 – 17 years of age) and adults (≥18 years of age), (2) parasite prevalence, (3) antigen source for immunoassay – recombinant protein versus gametocyte extract, (4) antigen coating concentration, and (5) seropositivity cut-off – two versus three standard deviations (SD) above the antibody reactivity of a malaria naïve population. Malaria naïve populations were either a non-exposed Caucasian population or a statistically-defined population of low responders. For the variable age, where a study used age categories that marginally overlapped the pre-defined age groups, for instance, children 0 – 19 years of age or adults >16 years of age, and data were not available to reanalyse the age groups, then the original study's age categories were used to define children and adults. Microscopy was the most common parasite detection method. Therefore, microscopy-based parasite prevalence estimates were used in the analyses.

To define transmission intensity at the time of sampling in a uniform way across all studies, I used data from Snow *et al.* (2017) on the changing parasite prevalence in Africa over time[419]. The authors collected data between the early 1900s and 2015 across different sites in Africa, spanning 520 sub-national administrative units. Using these data, the authors defined the predicted parasite rate standardised for 2 – 10-year olds ($Pf\text{PR}_{2\text{-}10}$) for each administrative unit. I used information provided in the manuscripts on the location (village, district or other administrative units) from which study participants were recruited to identify the corresponding administrative region for each study site. I then used previously defined endemicity cut-offs to categorise hypoendemic study sites; $Pf\text{PR}_{2\text{-}10} \leq 10\%$, mesoendemic study sites; $Pf\text{PR}_{2\text{-}10} > 10\%$ - 50%, or hyperendemic study sites $Pf\text{PR}_{2\text{-}10} > 50\%$.

Additionally, for longitudinal studies, seroprevalence estimates from separate cross-sectional surveys were combined to calculate a pooled seroprevalence estimate that was then used in the analyses. Where cross-sectional surveys were seasonally spaced, cross-sectional surveys carried out in the dry season or rainy season were considered separately in the univariable analysis during meta-regression. In cases where a study measured seroprevalence at the peak and at the end of the rainy season, the data were pooled and considered as seroprevalence measured during the rainy season. To compare seroprevalence to Pfs230 and Pfs48/45 reported in the different studies, a two-proportions Z-test was used. Where fewer than 5 participants were positive to either antigen, a Fisher's exact test was used.

2.5 Results

2.5.1 Flow diagram of studies retrieved for the review

A total of 525 articles were identified from the various databases during the literature search. After filtering out duplicate studies, 205 studies remained, and these were then screened by title and abstract. The screen identified 34 studies that contained relevant information. The full texts from these studies were evaluated and assessed against the inclusion criteria. After evaluation, 12 studies remained. These were then included in the systematic review and meta-analysis. A summary of the selection process is outlined in **Figure 2.1**.

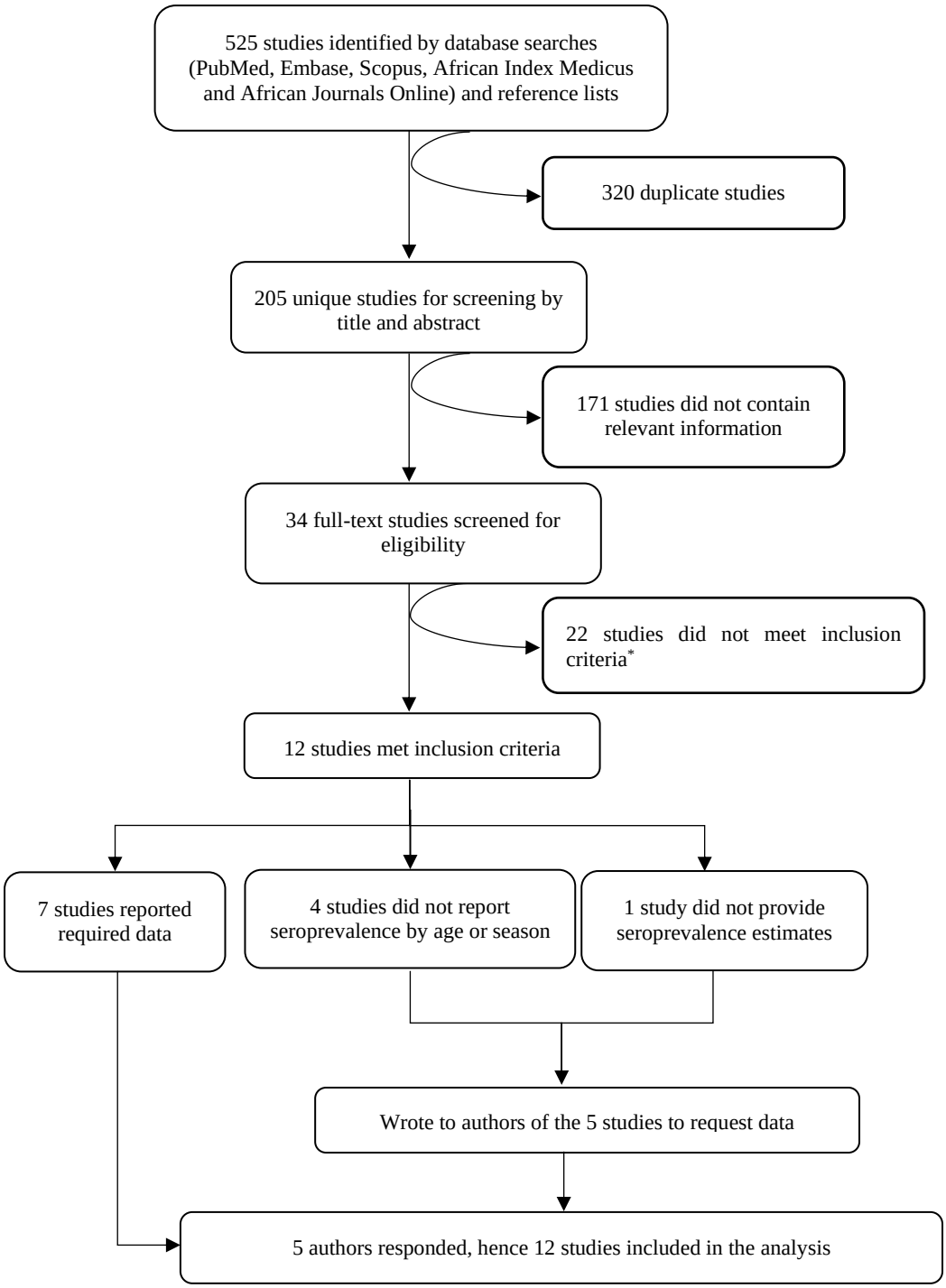


Figure 2.1: Consort diagram showing study selection for the systematic review and meta-analysis.
*****Reasons for exclusion included: 6 studies measured immune responses semi-quantitatively (4 of these in the same population); 11 studies had a small sample size (less than 30), and 5 studies were healthcare facility-based studies (primary care facilities and hospitals).

2.5.2 Study selection and characteristics

The 12 studies were from 21 locations in Africa that represented 17 study sites (as defined by the administrative region of the study site). The majority of the studies were carried out in West Africa (Burkina Faso, Senegal, Gabon, Cameroon, Ghana, and Mali), with only one study site in East Africa (Tanzania) and two study sites in Southern Africa (Zimbabwe) (**Table 2.1**). Most studies measured immune responses to both antigens; only two studies did not measure responses to Pfs230, and three studies did not measure responses to Pfs48/45. Additionally, six studies were longitudinal, with immune responses measured either in the dry season or in the rainy season. Children were the most common study population, and ELISA was the predominant immunoassay used.

Table 2.1: Characteristics of studies included in the systematic review and meta-analysis

Study (Reference)	Year	Country	Region of Study Site[c]	Sample Size	Age Group (years)	Antigen Detected	Seasonality Tested (Y/N)	Assay	Seropositivity Cut-off	Negative Control[d]
Amoah *et al.* [415 a]	2018	Ghana (Abura)	Central	65	6 - 12	Pfs230	No	ELISA^R	2 SD	Naïve
Amoah *et al.* [415 a]	2018	Ghana (Obom)	Greater Accra	75	6 - 12	Pfs230	No	ELISA^R	2 SD	Naïve
Lamptey *et al.* [228]	2018	Ghana	Greater Accra	338	2 - 65	Pfs230	Yes	ELISA^R	3 SD	Test sample
Stone *et al.* [74 b*]	2018a	Burkina Faso	Hauts-Bassins	33	5 - 14	Pfs230 and Pfs48/45	No	ELISA^R	3 SD	Test sample
Stone *et al.* [74 b*]	2018b	Burkina Faso	Centre-Nord	38	2 - 10	Pfs230 and Pfs48/45	No	ELISA^R	3 SD	Test sample
Stone *et al.* [74 b*]	2018	Cameroon	Centre	140	5 - 16	Pfs230 and Pfs48/45	No	ELISA^R	3 SD	Test sample
Bansal *et al.* [420]	2017	Zimbabwe	Mashonaland Central	181	6 - 14	Pfs48/45	No	ELISA^R	2 SD	Naïve
Paul *et al.* [220]	2016	Zimbabwe	Manicaland	150	6 - 16	Pfs48/45	No	ELISA^R	2 SD	Naïve
Ateba-Ngoa *et al.* [421 b]	2016	Gabon	Moyen - Ogooue	286	3 - 50	Pfs230 and Pfs48/45	No	ELISA^R	3 SD	Test sample
Jones *et al.* [203 b]	2015	Burkina Faso	Nord	200	5 - 16	Pfs230 and Pfs48/45	Yes	ELISA^R	3 SD	Test sample
Jones *et al.* [203 b]	2015	Ghana	Greater Accra	108	5 - 17	Pfs230 and Pfs48/45	Yes	ELISA^R	3 SD	Test sample

Study (Reference)	Year	Country	Region of Study Site[c]	Sample Size	Age Group (years)	Antigen Detected	Seasonality Tested (Y/N)	Assay	Seropositivity Cut-off	Negative Control[d]
Jones *et al.* [203 b]	2015	Tanzania	Tanga Region	202	3 - 15	Pfs230 and Pfs48/45	Yes	ELISA^{R}	3 SD	Test sample
Skinner *et al.* [227 b]	2015	Mali	Koulikoro 3 and Bamako	225	2 - 25	Pfs230 and Pfs48/45	Yes	Microarray^{R}	2 SD	No template
Miura *et al.*, [221]	2013	Mali	Kayes 2	45	18 - 60	Pfs230	No	ELISA^{R}	3 SD	Naïve
Ouedraogo *et al.* [200 b*]	2018	Burkina Faso	Centre-Nord	128	1 - 55	Pfs230 and Pfs48/45	Yes	Two-site ELISA^{Ge}	3 SD	Naïve
Ouedraogo *et al.* [217 a]	2011	Burkina Faso	Centre-Nord	296	1 - >20	Pfs230 and Pfs48/45	Yes	Two-site ELISA^{Ge}	2 SD	Naïve
Van der Kolk *et al.* [214]	2006	Cameroon	Centre	236	5 - 14	Pfs230 and Pfs48/45	No	Two-site ELISA^{Ge}	2 SD	Naïve

[a] Seroprevalence data requested from authors.
[b] Seroprevalence data calculated from data provided by original authors, or from data deposited on public repositories.
[b*]Citation includes repository from which data was retrieved.
[c] Administrative region of study site from which participants were drawn.
[d] Negative control refers to the comparator used to assign seropositivity. Naïve – malaria naïve volunteers; sample – a proportion of statistically-defined seronegative individuals; no template - a 'no DNA control' used to detect reactivity to the expression vector used to produce protein for the array.
^{R} Recombinant protein; ^{Ge} gametocyte extract.
SD – standard deviation.

2.5.3 Pfs230

2.5.3.1 Seroprevalence

Of the 12 studies selected for analysis, ten studies from across the 15 study sites in Africa analysed antibody (IgG) responses to Pfs230. There was a broad range of reported seroprevalence estimates to Pfs230, varying from 6% reported by Stone *et al.* (2018) in Soumousso and Dande villages, Burkina Faso[74] to 72% reported by Amoah *et al.* (2018) in Obom, Ghana[415] (**Figure 2.2**). Analysis of heterogeneity revealed significant between-study heterogeneity (I^2 = 97%; 95% CI: 96 – 98%; p < 0.01) preventing the calculation of a pooled seroprevalence estimate.

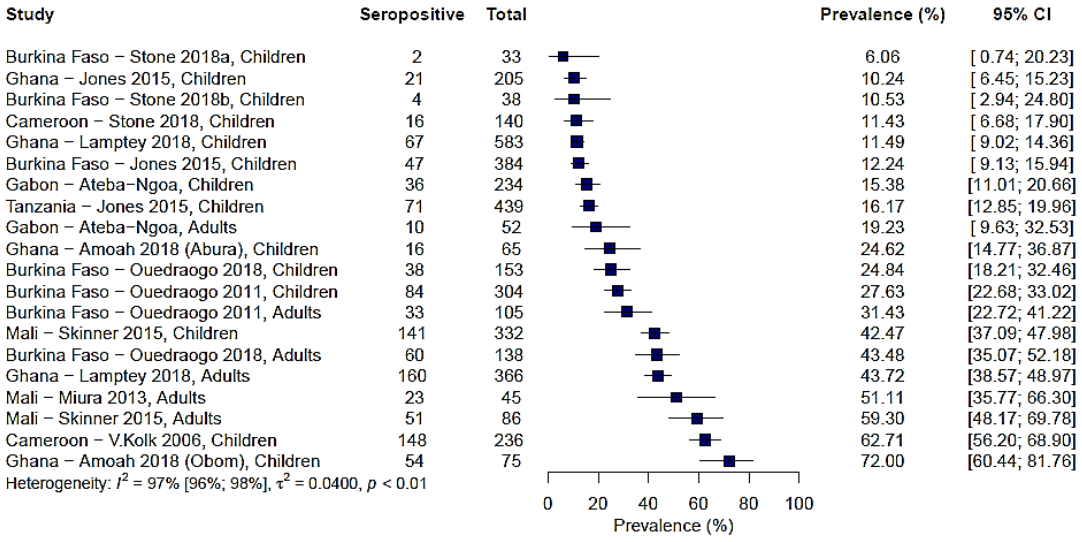


Figure 2.2: Forest plot of seroprevalence to Pfs230. Seropositivity was calculated based on a cut-off derived from either seronegative individuals or an assay negative control. Figure from Muthui *et al.* (2019)[422].

2.5.3.2 Factors associated with reported seroprevalence to Pfs230

To identify factors associated with seroprevalence to Pfs230, and in so doing also identify contributors to the heterogeneity observed, I performed a meta-regression analysis. I explored how differences relating to both the study population, as well as the immunoassay protocol employed, impacted the reported seroprevalence to Pfs230. In comparison to studies in children, studies in adults were associated with higher seroprevalence estimates (β coefficient 0.21, 95% CI: 0.05 – 0.38, p = 0.042) (**Table 2.2**). Unlike with participant age, higher asexual parasite and gametocyte prevalence

were not associated with seroprevalence estimates. Similarly, no significant association was seen between transmission intensity of the study site or sampling season (dry versus rainy) and seroprevalence to Pfs230.

When methodological factors were considered, the antigen source was significantly associated with estimates of seroprevalence to Pfs230. For studies that used recombinant protein, a higher antigen coating concentration of 1 µg/ml was associated with higher seroprevalence estimates in comparison to a coating concentration of 0.1 µg/ml (β coefficient 0.26, 95% CI: 0.09 – 0.43, p = 0.042). Moreover, the seropositivity cut-off was also significantly associated with seroprevalence estimates. A three-SD cut-off above the immune responses of seronegative individuals associated with lower seroprevalence estimates when compared to a 2 SD cut-off (β coefficient -0.22, 95% CI: -0.37 – -0.06, p = 0.042).

There was no association, however, between the source of antigen (gametocyte extract or recombinant protein) and seroprevalence estimates. Multivariable meta-regression to test the combined significance of the factors identified in the univariable analysis was not feasible owing to the small number of studies included, and the fact that not all variables were reported for each study.

While multivariable meta-regression analysis was not possible, I explored a two-variable analysis using age and seropositivity cut-off that were associated with seroprevalence estimates. Most studies reporting seroprevalence in adults used a 3 SD cut-off. However, the associations between older age and higher seroprevalence estimates do not indicate a bias in the observed associations. This was confirmed in the two-variable meta-regression analysis, where statistically significant associations between increased age and higher seroprevalence as well as the 3 SD cut-off and lower seroprevalence estimates were observed (**9.2 Appendix 2**). Though all included studies reported the seropositivity cut-off, only 6 studies used recombinant protein and reported the antigen coating concentration. A robust two-variable meta-regression could not therefore be carried out.

Additionally, I also analysed the amount of heterogeneity explained by each of the variables tested. However, none of the variables resulted in a reduction in heterogeneity to below 75%. The high heterogeneity was also exemplified by sub-

group analyses using age group, antigen coating concentration and seropositivity cut-off (**9.2 Appendix 2**)

Table 2.2: Univariable meta-regression analysis of factors influencing reported seroprevalence to Pfs230

Covariate	No. of Studies (No. of Sites)	Coefficient (β)	95% CI	*p*-value*	Residual I^2	I^2 Change (%)
Age						
Children (ref)	10 (14)	.	.	.	.	
Adults	6 (6)	0.21	0.05, 0.38	**0.04**	95.36	**2.09**
Asexual parasite prevalence	6 (10)	-0.001	-0.005, 0.002	0.51	95.37	2.08
Gametocyte prevalence	4 (8)	-0.002	-0.004, 0.001	0.38	92.54	4.50
Transmission intensity						
Mesoendemic (ref)	7 (8)	.	.	.	.	
Hyperendemic	6 (7)	-0.06	-0.23, 0.11	0.51	96.18	1.25
Season						
Dry (ref)	6 (9)	.	.	.	.	
Rainy	5 (7)	0.07	-0.12, 0.27	0.51	96.24	1.19
Antigen source						
Gametocyte extract (ref)	3 (3)	.	.	.	.	
Recombinant protein	7 (12)	-0.06	-0.25, 0.13	0.51	96.31	1.12
Antigen concentration[+]						
0.1 µg/ml (ref)	3 (7)	.	.	.	.	
1 µg/ml	3 (4)	0.26	0.09, 0.43	**0.04**	93.52	**3.98**
Seropositivity cut-off						
2 SD (ref)	4 (5)	.	.	.	.	
3 SD	6 (10)	-0.22	-0.37, -0.06	**0.04**	95.16	**2.30**

*p– values adjusted using the Benjamini and Hochberg correction for multiple testing; values in bold $p < 0.05$.
+Antigen concentration used as a variable for studies using recombinant protein as an antigen source.
CI – confidence interval, SD – standard deviation

2.5.4 Pfs48/45

2.5.4.1 Seroprevalence

There were nine studies carried out over 13 study sites that measured antibody (IgG) responses to Pfs48/45. Like Pfs230, there was a broad range in the reported seroprevalence to Pfs48/45 from as low as 0% from Stone *et al.* (2018) from study sites in Burkina Faso[74] to 64% reported by Paul *et al.* (2016) from their study in the Makoni district in Zimbabwe[220] (**Figure 2.3**). The between-study heterogeneity was I^2 96% (95% CI: 95% – 97%; $p < 0.01$) which again was above the 75% threshold, therefore, a pooled prevalence estimate was not calculated.

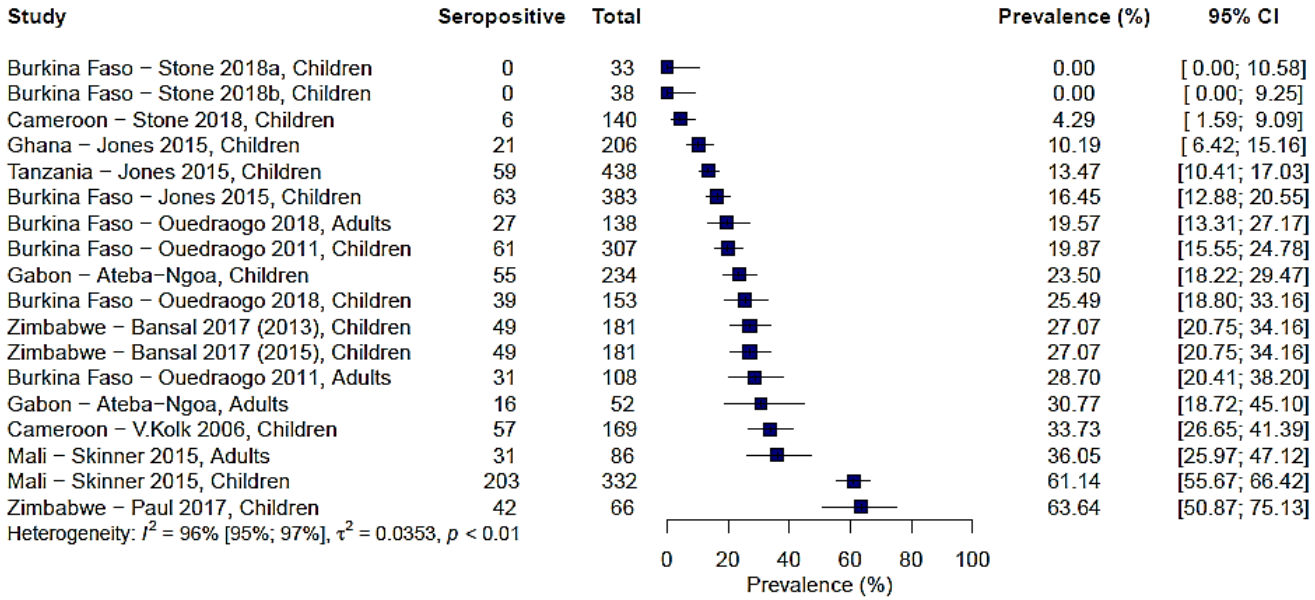


Figure 2.3: Forest plot of the seroprevalence to Pfs48/45. Seropositivity was calculated based on a set cut-off derived from either seronegative individuals or an assay negative control. Figure from Muthui *et al*. (2019)[422].

2.5.4.2 Factors associated with reported seroprevalence to Pfs48/45

I performed a meta-regression analysis to identify factors that were associated with seroprevalence to Pfs48/45. From the univariable analysis, age was not associated with seroprevalence estimates, unlike what was observed for Pfs230 (**Table 2.3**). There was a significant association between gametocyte prevalence and seroprevalence estimates, with higher gametocyte prevalence associated with lower seroprevalence to Pfs48/45 (β coefficient -0.003, 95% CI: -0.005 – -0.002, $p = 0.003$). No trend was seen for asexual parasite prevalence ($p = 0.11$). As with Pfs230, transmission intensity and sampling season were not significantly associated with seroprevalence to Pfs48/45.

For the methodological variables, as with Pfs230, higher antigen coating concentration (1 µg/ml) and a 3 SD cut-off were associated with lower seroprevalence estimates to Pfs48/45 (β coefficient 0.30, 95% CI: 0.06 – 0.54, $p = 0.043$ and β coefficient -0.26, 95% CI: -0.39 – -0.12, $p = 0.003$). Again, due to the limited number of studies, I did not attempt further multivariable analysis.

Table 2.3: Univariable meta-regression analysis of factors influencing reported seroprevalence to Pfs48/45

Covariate	No. of Studies (No. of Sites)	Coefficient (β)	95% CI	*p*-value*	Residual I^2	I^2 Change (%)
Age						
Children (ref)	9 (13)	.	.	.	.	
Adults	4 (4)	0.07	-0.12, 0.27	0.49	94.90	-0.18
Asexual parasite prevalence	4 (8)	-0.003	-0.006, 0.0003	0.11	91.41	3.96
Gametocyte prevalence	4 (8)	-0.003	-0.005, -0.002	**0.003**	70.82	**25.24**
Transmission intensity						
Hypoendemic (ref)	1(1)	.	.	.	.	
Mesoendemic	5 (6)	-0.47	-0.89, -0.06	0.11	93.91	0.87
Hyperendemic	5 (6)	-0.38	-0.8, 0.04			
Season						
Dry (ref)	4 (6)	.	.	.	.	
Rainy	6 (8)	0.07	-0.09, 0.24	0.47	93.12	1.70
Antigen						
Gametocyte extract (ref)	3 (3)	.	.	.	.	
Recombinant protein	6 (10)	-0.01	-0.19, 0.17	0.91	94.91	-0.19
Antigen concentration[ι]						
0.1 µg/ml (ref)	3 (7)	.	.	.	.	
1 µg/ml	2 (2)	0.30	0.06, 0.54	**0.043**	92.65	**2.20**
Seropositivity cut-off						
2 SD (ref)	5 (5)	.	.	.	.	
3 SD	4 (8)	-0.26	-0.39, -0.12	**0.003**	91.38	**3.54**

*p – values adjusted using the Benjamini and Hochberg correction for multiple testing; values in bold $p < 0.05$.
[+]Antigen concentration used as a variable for studies using recombinant protein as an antigen source.
CI – confidence interval, SD – standard deviation.

Of the variables tested, gametocyte prevalence appeared to explain a high degree of heterogeneity, resulting in a 25% reduction in the observed heterogeneity. Consequently, I decided to carry out subgroup analysis using data from the studies that reported gametocyte prevalence. I coded gametocytaemia as a categorical variable, grouping the prevalence into categories of less than 10%, 10% - 50%, and greater than 50%. The subgroup analysis revealed that the observed lower seroprevalence to Pfs48/45 at higher gametocyte prevalence was highly influenced by the study by Stone *et al.* (2018) where despite a majority of the children being gametocyte positive, they reported very low seroprevalence to Pfs48/45 (**Figure 2.4**). This was further confirmed by carrying out sensitivity analysis where the meta-regression was repeated in the absence of the Stone *et al.* (2018) study (**Table 2.4**). Gametocyte prevalence was now no longer associated with seroprevalence to Pfs48/45 (β coefficient 0.004, 95% CI: 0.000, 0.007, $p = 0.11$). Therefore, it was not possible to accurately define the nature of the relationship between gametocyte prevalence and seroprevalence to Pfs48/45 from the studies included in this analysis.

Furthermore, while heterogeneity was reduced to below 75% in the below 10% and 10 – 50% categories, only three studies were included (of the original nine studies). As a pooled seroprevalence estimate from the three studies disregards estimates from the 6 other studies, providing a pooled estimate of the seroprevalence to Pfs48/45 remained challenging. Additionally, though the antigen coating concentration, and seropositivity cut-off were statistically significant predictors in the meta-regression (**Table 2.4**), these variables did not result in a substantial reduction in the observed heterogeneity (**9.2 Appendix 2**).

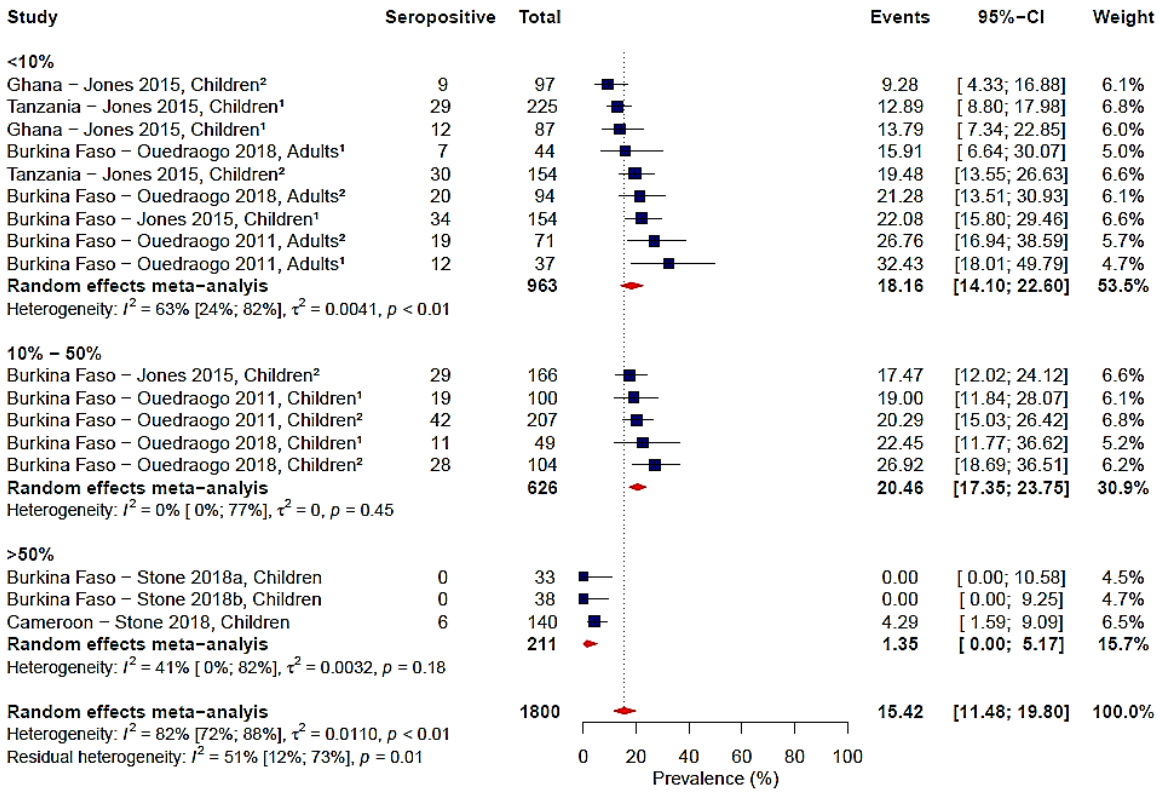


Figure 2.4: Forest plot of the seroprevalence to Pfs48/45 categorised by gametocyte prevalence. Seropositivity was calculated based on a set cut-off defined from either seronegative individuals or an assay negative control. [1]participants sampled during the dry season; [2]participants sampled during the rainy season. Figure from Muthui *et al*. (2019)[422].

Table 2.4: Univariable meta-regression analysis of factors influencing reported seroprevalence to Pfs48/45

Covariate	No. of Studies (No. of Sites)	Coefficient (β)	95% CI	*p-value**	Residual I^2 (%)	I^2 Change (%)
Age						
Children (ref.)	8 (10)	.	.	.	.	
Adults	4 (4)	0.01	-0.15, 0.18	0.97	92.88	-0.45
Asexual parasite prevalence	3 (6)	0.000	-0.004, 0.004	0.99	90.06	2.56
Gametocyte prevalence	3 (5)	0.004	0, 0.007	0.11	32.97	**64.34**
Transmission intensity						
Hypoendemic (ref.)	1 (1)	.	.	.	.	
Mesoendemic	4 (5)	-0.35	-0.71, 0.02	0.11	91.73	0.79
Hyperendemic	4 (4)	-0.43	-0.79, -0.06			
Season						
Dry (ref.)	4 (6)	.	.	.	.	
Rainy	6 (8)	0.07	-0.09, 0.24	0.47	93.12	-0.71
Antigen						
Gametocyte extract (ref.)	3 (3)	.	.	.	.	
Recombinant protein	4 (7)	0.08	-0.09, 0.25	0.47	93.12	-0.71
Antigen concentration[+]						
0.1 µg/ml (ref.)	1 (4)	.	.	.	.	
1 µg/ml	2 (2)	0.22	0.03, 0.41	0.09	87.41	**5.46**
Seropositivity cut-off						
2 SD (ref.)	5 (5)	.	.	.	.	
3 SD	3 (5)	-0.22	-0.35, -0.08	**0.027**	89.43	**3.28**

[*] *p* – values adjusted using the Benjamini and Hochberg correction for multiple testing; values in bold $p < 0.05$.
[+]Antigen concentration used as a variable for studies using recombinant protein as an antigen source.
CI – confidence interval, SD – standard deviation.

2.5.5 Pfs230 and Pfs48/45 Combined Seroprevalence

I then went on to perform a pairwise comparison of seroprevalence estimates for Pfs230 and Pfs48/45 using data from studies that analysed antibody responses to both antigens. I did this to determine if one antigen is more commonly recognised than the other. For this analysis, the seroprevalence was assayed in the same study population and using the same assay protocol per study. From the results, it did not appear that immune responses were consistently higher for either antigen. Some studies reported higher seroprevalence to Pfs230, while others reported higher seroprevalence to Pfs48/45 (**Figure 2.5**).

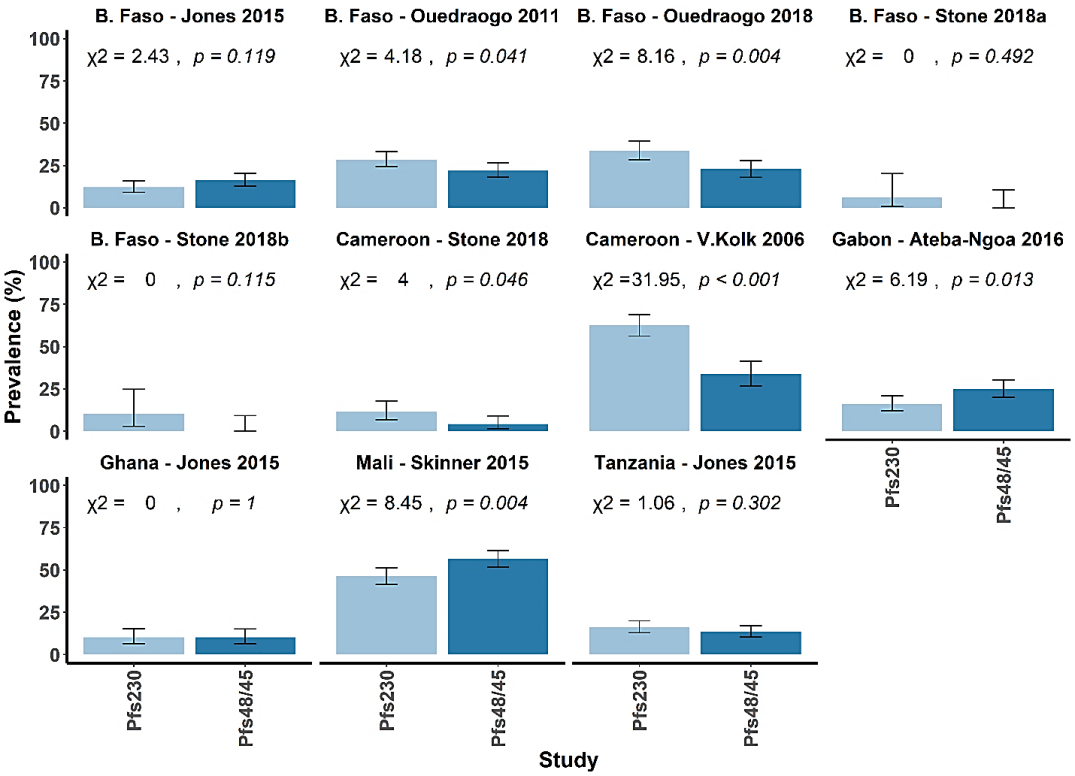


Figure 2.5: Comparison of seroprevalence to Pfs230 and Pfs48/45 in select studies. Seropositivity was calculated based on a set cut-off defined from either seronegative individuals or an assay negative control. Comparisons between proportions were carried out using a 2-proportions Z-test exception for the data from Burkina Faso for Stone *et al*. 2018a and 2018b where a Fisher's exact test was used owing to frequencies of below 5. Results from the tests (X^2 -where appropriate and *p* value) are presented in the graph. Error bars represent 95% confidence intervals. B. Faso – Burkina Faso.

2.6 Discussion

Calls to intensify malaria elimination efforts have renewed interest in developing interventions that interrupt transmission. These interventions include vaccines that interrupt malaria transmission (VIMTs) such as the classical transmission-blocking vaccines[5]. For the successful design, evaluation, and implementation of transmission-blocking vaccines, an improved understanding of naturally acquired immune responses to the transmissible parasite stages will be required. I therefore carried out a systematic review and meta-analysis of studies that looked at NAI to the lead vaccine candidate antigens Pfs230 and Pfs48/45 in African populations. In so doing, I aimed to estimate the seroprevalence to these two antigens by pooling studies and to understand the factors associated with seroprevalence to these two antigens at the population level. I focused on classical, well characterised, indicators of parasite exposure such as age, transmission intensity, season, and parasite prevalence as potential explanations of heterogeneity.

The reported seroprevalence to Pfs230 ranged from 6%[203] to 72% [415] and from 0% [74] to 64%[220] for Pfs48/45. This broad range reflected substantial between-study heterogeneity that precluded carrying out a pooled analysis to arrive at a single, reliable estimate of seroprevalence to either antigen. As a result, I sought to look at possible factors contributing to this heterogeneity using both the indicators mentioned above relating to malaria exposure and also methodological variables.

There has been much debate as to whether NAI to sexual stage antigens increases with age with some studies showing no association with age[217,218,423] while other studies demonstrate increasing antibody prevalence with age[74,200]. An increase in seroprevalence with age has been described for asexual stage antigens such as the merozoite surface proteins[155,184] or the infected erythrocyte protein family *Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP1)[412]. This gradual acquisition of immunity to clinical disease may reflect the time taken to acquire long-lived plasma cells and memory B cells following repeated parasite exposure[22,24.] Additionally, it may also indicate the time required to obtain a repertoire of antibodies to clonally variant antigens such as PfEMP1[25].

In this meta-analysis, a modest association was found between age and seroprevalence to Pfs230 but not to Pfs48/45. While antigens may differ in their ability to induce

long-lived immune responses, the lack of association seen with Pfs48/45 may also be attributable to the limited number of studies that analysed both children and adults as well as between-study heterogeneity. From this analysis, a firm conclusion cannot be made on the ability of Pfs48/45 to induce long-lived immune responses. Studies that have shown no association between immune responses to sexual stage antigens and age have argued that such immune responses reflect recent exposure rather than cumulative exposure[217,218]. While not conclusive, the results of this meta-analysis are evidence for the existence of long-lived immune responses to Pfs230. In support of this, Ouedraogo *et al.* (2018) found that seroprevalence and density of antibodies to Pfs230 and Pfs48/45 increased with age and this increase positively correlated with higher TBA[200]. Such an age-dependent response would suggest that not only immune recognition but also functional antibodies against the two antigens increase with age.

While individual studies showed an increase in seroprevalence to Pfs230 and Pfs48/45 during the rainy season[200,203,217], the combined analysis did not find a definitive association between sampling season and seroprevalence to the two antigens. The inability to observe an association between sampling season and seroprevalence estimates in the combined analysis, as in the individual studies, may indicate potential confounding factors that would need to be accounted for in multivariable analysis. Unfortunately, only six studies reported the sampling season, and this precluded further multivariable analysis. The potential for natural boosting of responses following natural parasite exposure during the rainy season favours the prioritisation of pre-fertilisation antigens such as Pfs230 and Pfs48/45 for TBV design[227,424,425]. In their study, Ouedraogo *et al.* (2018) found decreased infectiousness to mosquitoes during the malaria transmission season that coincided with boosted natural responses to Pfs230 and Pfs48/45[200].

Additional factors that I investigated concerning seroprevalence to Pfs230 and Pfs48/45 were transmission setting and parasite prevalence. While some studies demonstrated an increase in seroprevalence estimates in higher transmission settings, this was not replicated in the meta-analysis. Likewise, a definitive association between either asexual parasite prevalence or gametocyte prevalence and seroprevalence to either of the two antigens under investigation was not observed.

None of the variables related to malaria exposure described in the above paragraphs appreciably reduced the between-study heterogeneity; therefore, I decided to consider the contribution of methodological variability. Aspects in which the studies differed included the source of antigen for the immunoassay, choice of immunoassay, assay protocol, and seropositivity cut-off. Owing to the challenges in producing correctly-folded Pfs230 and Pfs48/45, early studies relied on whole antigen from gametocyte extract for their immunoassays[217,218,300,426]. These studies employed a two-site ELISA where epitope-targeted monoclonal antibodies were used for antigen capture before the addition of diluted human serum for antibody detection[217,218] or competition with a second monoclonal antibody[300]. Two-site ELISAs are reportedly less sensitive, resulting from either high background reactivity[203] or the fact that the epitope bound by the monoclonal antibody is inaccessible to the test antibodies[425]. The limited sensitivity may bias towards lower seroprevalence estimates reported in comparison to studies using ELISA or microarrays.

In this analysis, I did not find evidence of antigen source influencing seroprevalence estimates. Additionally, as the same studies that used gametocyte extract also used a two-site ELISA and only one study used the microarray platform, I could not evaluate the contribution of immunoassay to methodological variability. For antigen concentration, studies using a lower antigen concentration for the immunoassays reported lower seroprevalence estimates. For this reason, it is paramount that studies optimise their antigen coating concentration and antibody dilutions to allow better distinction of seropositive and seronegative individuals. Moreover, the seropositivity cut-off used to distinguish seropositives from seronegatives was associated with seroprevalence estimates. Typically, seropositivity is estimated by defining a cut-off based on two or three standard deviations (SD) from the mean response in seronegative individuals. This analysis found that a higher cut-off of 3 SD was associated with lower seroprevalence estimates, reflecting the higher stringency.

The significant influence of methodological variables on seroprevalence estimates makes a case for establishing a ‘gold standard’ set of criteria that should be adopted when analysing seropositivity to sexual stage antigens. These criteria could include (1) use of appropriately folded and validated recombinant proteins where possible (with indications of the protein expression system used and protein region targeted), (2) use of a 3 SD cut-off for seropositivity estimation, and (3) reporting of antigen

coating concentration and serum dilution used in an immunoassay. Additionally, for reporting of the results of seroepidemiological studies on sexual stage antigens, the Malaria Immunoepidemiology Observational Studies (MIOS) guidelines proposed by Fowkes *et al.* (2010)[427] should be adopted. Clear guidelines would allow reproducibility, standardisation, and possibly generalisability of findings from seroepidemiological studies.

2.6.1 Limitations

The majority of the studies used microscopy as the parasite detection method; hence I was limited to using microscopy-based estimates of parasite prevalence. The limited sensitivity of microscopy for parasite detection has been well described[428,429] and tends to underestimate parasite prevalence. Molecular-based methods of parasite detection may offer a better understanding of the relationship between parasite prevalence and seroprevalence to sexual stage antigens thus providing an improved understanding of the dynamics of NAI in the context of sexual stage immunity.

Additionally, few studies looking at naturally acquired immunity to gametocyte antigens were identified for inclusion in the analyses, potentially limiting the generalisability of these findings. The limited number of studies, and the fact that not all studies reported information on the variables investigated, meant that further examination of the sources of heterogeneity identified in the univariable analysis could not be robustly evaluated in multivariable analysis. The high heterogeneity between studies arising from methodological differences complicated efforts to calculate pooled estimates. Standardised protocols for conducting and reporting seroepidemiological studies are therefore required. Reporting pooled estimates across studies may still present a challenge as not all variables can be standardised between studies, for example, transmission intensity. However, standardised protocols would allow a better estimation of the actual heterogeneity in reported estimates.

2.6.2 Summary of overall findings

This systematic review and meta-analysis revealed that antibody responses to the sexual stage antigens Pfs230 and Pfs48/45 are prevalent in the populations studied, with a broad range of seroprevalence estimates reported. Of the variables investigated for a potential relationship with seroprevalence, I found evidence for a role for age as a determinant of seroprevalence to Pfs230. This would suggest that long-lived, stable

responses to sexual stage antigens may develop in malaria-endemic populations and may provide additional criteria for screening newly identified TBV candidates. Additionally, the potential for boosting of vaccine-induced responses following the malaria transmission season may enhance vaccine efficacy in the field.

The high heterogeneity between studies arising from methodological differences complicated efforts to calculate pooled estimates. More standardised methods for conducting and reporting seroepidemiological studies would facilitate comparison between different studies.

Chapter 3

Describing the prevalence and distribution of gametocytaemia among children in the Kilifi Malaria Longitudinal Rolling Cohort

3.1 Introduction

Heterogeneity of malaria transmission is a well-described phenomenon where certain individuals are more likely to get infected and consequently more likely to transmit malaria[430–433]. Targeted application of malaria control tools would, therefore, be highly beneficial in curbing transmission in such hotspots. This would require an improved understanding of individuals who contribute disproportionately to transmission, and the identification of prognostic indicators that could identify such individuals. Not only would this aid the effective implementation of existing malaria control tools, but it would also assist the design of improved transmission-blocking interventions[434].

For transmission of malaria, a mosquito needs to take up male and female gametocytes during a blood meal. Gametocyte production is a 'dead-end' for replication, thus complicating within-host survival[28]. Therefore, the parasite relies on specific cues to determine whether to continue replicating asexually, thus maximising within-host survival, or commit to the sexual development pathway and ensure between-host transmission[75]. Consequently, commitment to gametocytogenesis and subsequent gametocyte carriage is likely to vary between individuals in response to host factors that alter parasite fitness. The precise mechanisms governing gametocyte commitment are not yet well described. However, environmental cues appear essential in determining the level of investment to gametocytogenesis during an infection[75,435]. Factors such as drug pressure, an unfavourable host environment and host immune factors[32,77,78] are thought to play a role in modulating gametocyte production.

Concerning the link between drug pressure and gametocyte carriage, the nature of the association observed depends on the type of antimalarial drug used. With older antimalarials, such as chloroquine (CQ) and sulphadoxine-pyrimethamine (SP), an increase in post-treatment gametocyte carriage has been described[67,436–438]. Further, this effect was enhanced when resistance emerged[437,439]. The current first-line drugs for the treatment of malaria are the artemisinin combination therapies (ACTs). While

ACTs are not active against mature circulating gametocytes, but they are active against immature gametocytes thereby reducing gametocyte carriage[433,434]. Some data show, however, that the impact of ACTs on gametocyte carriage depends on the type of ACT used. Combinations of artemether-lumefantrine and artesunate-mefloquine reportedly show more significant reductions in gametocyte burden than either dihydroartemisinin-piperaquine or artesunate-amodiaquine[104]. This is likely due to the dosing regimen or activity of the partner drug[104]. The gametocidal properties of different antimalarials are, therefore, an essential factor to consider when looking into factors that affect post-treatment gametocyte carriage.

In addition to the environmental stressors described above (drug pressure and an unfavourable host environment), host genetics may also influence gametocyte production. The effect of haemoglobinopathies, particularly sickle cell trait (haemoglobin (Hb) S), haemoglobin C disease (HbC) and α-thalassaemia, has been evaluated in the context of malaria where these variants have been shown to confer protection against severe disease[128–130]. On the other hand, these RBC disorders have been linked to increased gametocyte carriage[68,79]. This may occur due to the anaemia-induced increase in immature RBCs or reticulocytes[441,442]. Reasons why gametocytes prefer reticulocytes could include molecular or biochemical properties of the reticulocytes[443]. An alternate explanation is that the development time of reticulocytes in the bone marrow coincides with the time taken for gametocytes to mature[443].

As with the haemoglobinopathies, ABO blood groups may also protect against severe malaria. Relative to AB and A blood groups, blood group B and more so blood group O reduce parasite rosetting, thus limiting the severity of disease[138,139]. In their study, Grange *et al*. (2015) found a case for increased gametocyte carriage linked to B and O blood groups[68]. A mechanism for this association, however, has not yet been described. The range of factors that influence gametocyte carriage raises the possibility of identifying key prognostic features that could indicate individuals at a higher risk of gametocyte carriage. These features could then serve to guide the implementation of transmission-blocking interventions maximising their impact.

3.2 Rationale

While there have been studies looking into the epidemiology of gametocyte carriage, these have primarily been single surveys or longitudinal studies with relatively short follow-up [69,436,444–447]. Few studies have employed more extended periods of follow-up[68,448,449]. I had access to a unique dataset from a longitudinal cohort of children followed up cumulatively for over 19 (from 1998 to 2016) years to track parasite prevalence along the Kenyan coast. The Kilifi Malaria Longitudinal Rolling Cohort (KMLRC) presented an opportunity to adequately describe, characterise and explore potential factors influencing gametocyte carriage. Over time, malaria transmission patterns have changed dramatically in this region, as has malaria drug use, providing an opportunity to investigate how this has affected gametocyte carriage. I thus used this dataset to better understand and describe the prevalence and distribution of gametocytaemia, and also potentially identify risk factors for gametocyte carriage.

To have a comprehensive view, I used data from cross-sectional surveys done to assess asymptomatic parasite carriage as well as weekly-surveillance data collected to track clinical cases of malaria. This analysis was done to both gain better insights into the patterns of gametocyte carriage in the KMLRC and inform the seroepidemiological studies of responses to gametocyte antigens (**Chapter 5**). Insights gleaned from this chapter, combined with findings on the factors influencing naturally acquired sexual stage immune responses (**Chapter 2**) and the seroepidemiological studies, aimed to provide a better understanding of the dynamics of transmission-blocking immunity. I assessed likely prognostic indicators of gametocytaemia as these would be important in determining the factors that influence the prevalence of antibodies to gametocyte antigens. Furthermore, the analysis provided an opportunity to identify individuals positive for gametocytes, and appropriate controls, that would serve the basis for immunoprofiling responses to the candidate gametocyte antigens.

3.3 Objectives

The main objective was to describe the prevalence and distribution of gametocytaemia among children in a longitudinal rolling cohort in Coastal Kenya using data collected over 19 years of follow-up.

3.3.1 Specific Objectives

- Describe key factors that are associated with gametocyte carriage.
- Identify gametocyte carriers and appropriate controls for seroepidemiological analysis of naturally acquired immune responses to gametocyte antigens.

3.4 Methods

3.4.1 Study design and data collection

The KMLRC, from where study participants were recruited, is located in Kilifi County, within the Kilifi Health and Demographic Surveillance System (KHDSS), along the Kenyan coast (**Figure 3.1**)[450–452]. Three cohorts with varied transmission intensity were included, being: Ngerenya (initially of moderate transmission but declining to low transmission), Junju (moderate transmission), and Chonyi (high transmission). Peak malaria transmission occurs during the rainy seasons with the long rainy season occurring between May – July and the short rainy season between October – December[450,453].

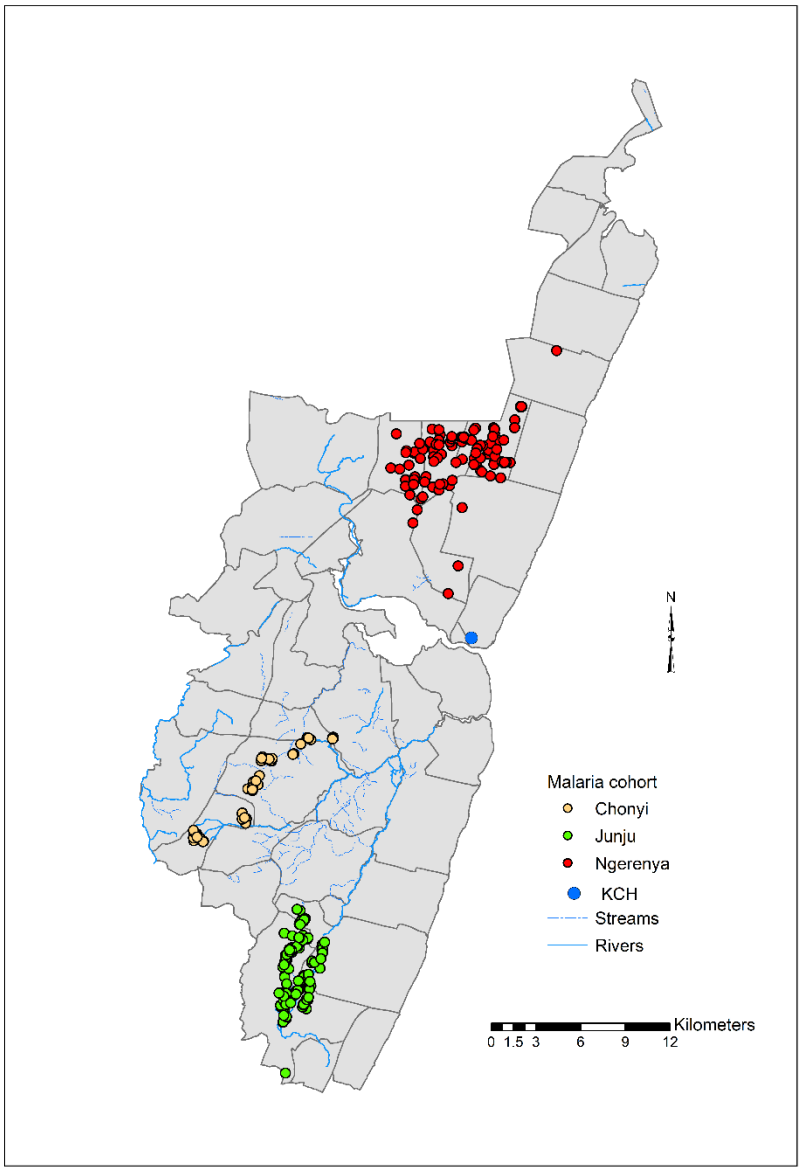


Figure 3.1: Map of the Kilifi Malaria Longitudinal Cohort located within the Kilifi Health and Demographic Surveillance System (KHDSS). Participants homesteads within the cohort are indicated by coloured points. Chonyi (orange); Junju (green); and Ngerenya (red). KCH, Kilifi County Hospital. Figure from Muthui *et al.* (2019)[454].

I analysed cross-sectional survey data from the three cohorts, with cross-sectional surveys conducted from 1998 to 2016 for Ngerenya (no survey was conducted in 2006); from 1999 to 2001 for Chonyi; and from 2007 to 2016 for Junju. In addition to the cross-sectional surveys that aimed to detect asymptomatic parasite carriage, children were actively monitored by field workers each week to identify any episodes of febrile malaria. Over the analysis period, there was a marked decrease in malaria transmission intensity in Ngerenya[455] that necessitated dividing the cohort into two time-periods for analytical purposes. Ngerenya was thus divided into Ngerenya early

(1998 – 2001) a period of moderate transmission, and Ngerenya late (2002 – 2016) a period of moderate to low transmission based on the drop in parasite prevalence observed after 2001 (**Figure 3.2**). From this point onwards, the KMLRC will be considered to have four cohorts.

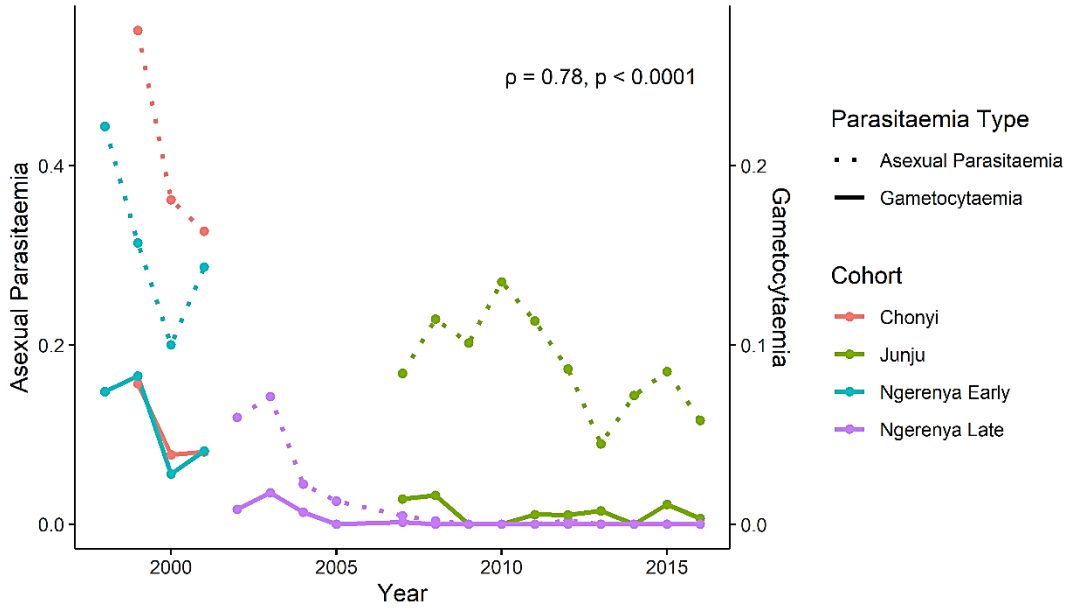


Figure 3.2: Parasite prevalence over time. Line plot depicting the temporal variation in microscopically-determined *P. falciparum* parasite prevalence over time. The calculated spearman's rank correlation co-efficient of the variation in asexual and gametocyte prevalence over time is also provided in the graph.

Study recruitment began in 1998, with households selected at random from Ngerenya and Chonyi locations[450,451]. Seventy-two households were chosen in Ngerenya (819 participants) and 52 households in Chonyi (783 participants). There was no official calculation of sample size, though the number of participants was considered adequate to study the clinical definitions of malaria. Children aged 15 years and below were recruited at the start of the study (age distribution over time is provided in **9.3Appendix 3**). Recruitment for Junju cohort was from 405 children belonging to 149 homesteads, aged between one to six years, who had previously participated in a malaria vaccine trial[452]. These children and their siblings were subsequently recruited for longitudinal monitoring. The sample size of 400 children allowed the detection of 35% vaccine efficacy with 80% power based on an anticipated 50% malaria incidence rate. Subsequently, children born into these households were recruited to join the

longitudinal cohort and were followed up until the age of 15 when they exited the study[129]. Each respective year, the same study protocol was applied across the three cohorts, and all participants had equal access to healthcare facilities.

3.4.2 Ethics approval and consent to participate

Ethical approval for participation in the KMLRC study was given by the Kenya Medical Research Institute Ethics Review Committee (reference numbers KEMRI/SERU/CGMRC//3149 and SSC1131). All research was conducted according to the principles of the Declaration of Helsinki, which included consenting participants in their local language before any study procedure was conducted. Parents of the children involved in the KMLRC study provided written informed consent for participation in this study.

3.4.3 Malaria case detection

Active case detection for malaria was carried out during weekly follow-up visits by field workers as previously described[450,456]. Briefly, each week households in the three cohorts were visited by a field worker, and axillary temperature recorded for each study participant. Blood smears were performed for parasite detection if a participant presented with fever or reported having fever in the days leading up to the visit. Rapid diagnostic tests (RDTs – Diamed OptiMAL parasite lactate dehydrogenase (pLDH test), which was eventually replaced with CareStart™ Malaria *Pf* (HRP2)) were available for active case detection in the field from 2007 onwards and were used to guide treatment decisions. However, even before RDT introduction, all febrile malaria episodes were treated. Free treatment was readily available to the study participants as the field workers were resident in the villages from where study participants were recruited and were on hand to assess febrile episodes arising before a scheduled visit. National guidelines for the treatment of malaria provided by the Government of Kenya were followed and dictated the anti-malarial drug administered in a particular year.

3.4.4 Cross-sectional parasitological surveys

Cross-sectional parasitological surveys were undertaken to assess parasite prevalence before the onset of the long rainy season to determine asymptomatic *P. falciparum* infections. Approximately 363 (range 139 – 556) participants participated in each survey. A summary of the cross-sectional surveys included in the analysis is provided in **Table 3.1** with a breakdown of participants attending each survey**.**

Table 3.1: Summary of cross-sectional parasitological surveys carried out in the Kilifi Malaria Longitudinal Cohort between 1998 - 2016

Cohort	Cross-sectional survey number	Dates		Number of participants	Time of bleed
		Start date	End date		
Ngerenya early	1	31-08-98	11-09-98	419	In the dry season
Ngerenya early	2	12-07-99	18-07-99	532	During rains
Ngerenya early	3	13-03-00	18-03-00	537	Before long rains
Ngerenya early	4	12-07-00	15-07-00	515	During rains
Ngerenya early	5	03-10-00	26-10-00	556	Before short rains
Ngerenya early	6	19-03-01	30-03-01	555	Before long rains
Ngerenya early	7	13-06-01	16-06-01	522	During rains
Ngerenya late	1	13-05-02	28-06-02	309	Before long rains
Ngerenya late	2	11-10-02	27-11-02	295	Before short rains
Ngerenya late	3	12-05-03	23-05-03	295	Before long rains
Ngerenya late	4	21-10-03	09-02-04	298	Before short rains
Ngerenya late	5	26-04-04	16-06-04	297	Before long rains
Ngerenya late	6	18-10-04	09-12-04	283	Before short rains
Ngerenya late	7	16-05-05	04-06-05	270	Before long rains
Ngerenya late	8	02-05-07	10-05-07	268	Before long rains
Ngerenya late	9	22-08-07	29-08-07	285	In the dry season
Ngerenya late	10	26-11-07	30-11-07	291	Before short rains
Ngerenya late	11	04-02-08	07-02-08	277	Before long rains
Ngerenya late	12	14-05-08	21-05-08	279	Before long rains
Ngerenya late	13	20-04-09	12-05-09	274	Before long rains
Ngerenya late	14	03-05-10	07-05-10	267	Before long rains
Ngerenya late	15	25-05-11	31-05-11	264	Before long rains

Cohort	Cross-sectional survey number	Dates		Number of participants	Time of bleed
		Start date	End date		
Ngerenya late	16	02-04-12	10-04-12	262	Before long rains
Ngerenya late	17	23-04-13	29-04-13	243	Before long rains
Ngerenya late	18	31-03-14	17-04-14	215	Before long rains
Ngerenya late	19	28-04-15	05-05-15	150	Before long rains
Ngerenya late	20	30-03-16	04-04-16	139	Before long rains
Chonyi	1	12-07-99	17-07-99	510	During rains
Chonyi	2	13-03-00	18-03-00	532	Before long rains
Chonyi	3	10-07-00	15-07-00	501	During rains
Chonyi	4	11-10-00	27-10-00	516	Before short rains
Chonyi	5	19-03-01	28-03-01	528	Before long rains
Chonyi	6	11-06-01	16-06-01	515	During rains
Junju	1	11-05-07	23-05-07	339	Before long rains
Junju	2	11-08-07	22-08-07	364	In the dry season
Junju	3	12-11-07	19-11-07	360	Before short rains
Junju	4	04-02-08	07-02-08	340	Before long rains
Junju	5	05-05-08	13-05-08	346	Before long rains
Junju	6	27-04-09	14-05-09	361	Before long rains
Junju	7	11-05-10	21-05-10	377	Before long rains
Junju	8	16-05-11	24-05-11	377	Before long rains
Junju	9	11-04-12	20-04-12	400	Before long rains
Junju	10	02-04-13	17-04-13	411	Before long rains
Junju	11	28-04-14	15-05-14	392	Before long rains
Junju	12	13-04-15	27-04-15	378	Before long rains
Junju	13	14-03-16	24-03-16	316	Before long rains

3.4.5 Laboratory investigations

Blood films, both thick and thin, were taken from all study participants at the cross-sectional surveys and for participants presenting with fever during the weekly surveillance. For parasitological examination, the thin blood films were fixed with 100% methanol and stained with 3% Giemsa stain for 45 minutes, while thick films were air-dried before staining. Thick films were used for parasite counts unless more than 25 parasites were observed per high powered field then the thin film was used. If the full blood count was available, this was used to estimate the final parasitaemia. If not, a WBC count of 8×10^9 per litre or an RBC count of 5×10^{12} per litre was used. In a comparison between the use of full or estimated blood counts, no significant difference in parasitaemia estimated using either method was found[450]. One hundred high-powered fields of a thick film were read before confirming that no parasites were present. The microscopy protocol was designed to assess asexual parasitaemia primarily, and hence gametocytes were counted when observed during assessment for asexual parasitaemia. Therefore, the number of fields observed where gametocytes might be detected varied according to the asexual parasitaemia.

Two independent readers determined the parasite counts with discrepant readings resolved by a third reader. Over the study period, quality assurance involved comprehensive microscopy training as well as the use of internal and external quality control measures. Internal quality control involved using a subset of slides randomly selected each quarter for reassessment. Concordance between the two readings was then evaluated. The external quality control varied over the study period. Initially, reference blood films obtained from a partner lab in the United Kingdom were used to assess the proficiency of the microscopists. Currently, external quality control involves participating in quarterly evaluations by the National Institute of Communicable Diseases (NICD) based in South Africa where 20 slides per survey are sent from the NICD to our lab for proficiency testing.

In addition to parasitaemia, information on sickle cell genotype and α-thalassaemia status for a subset of study participants were assessed using previously described methods[457,458].

3.4.6 Case definitions

Data collected from the weekly follow-up visits was used to determine malaria episodes as previously described[450]. Axillary temperature was recorded at each visit and where fever was detected, a blood-film was taken for parasite assessment as described in 3.4.5. For children under one year of age, a malaria episode was defined as a fever with any parasitaemia[450]. For older children (1 – 15 years of age), a more conservative cut-off of fever with a parasite density of ≥2,500 parasites/µl of blood was used[450]. Malaria episodes were considered distinct if they occurred ≥ 28 days after the previous episode. A summary of the episodes that were pooled by each cohort is provided in **9.3 Appendix 3**. The sum of the malaria episodes that occurred in the interval between the respective survey (survey x) and the prior survey (survey x-1) then formed the number of malaria episodes occurring in the period leading up to each survey. The total number of malaria episodes for a survey period was corrected for the interval (days) between surveys.

3.4.7 Statistical analysis

Spearman's rank correlation was used to assess the relationship between continuous variables. Pairwise proportions tests between age groups were carried out using Chi-square or Fisher's exact tests (where sample sizes were below 5) with Bonferroni correction for multiple testing. Furthermore, models were fitted to determine the factors associated with gametocyte positivity. Initially, Poisson and logistic regression models were evaluated before determining the model that best fit the data comparing the Akaike information criterion (AIC). Based on the results a logistic regression model was chosen for the analysis. Variables used as covariates in the models included: asexual parasite positivity, age, year, number of malaria episodes and whether the participant had a malaria episode (as defined in **3.4.6**), asexual parasite positive blood film or gametocyte positive blood film in the prior cross-sectional survey. These variables were chosen as they have previously been shown to be associated with gametocyte carriage[77,459]. Age was converted to a categorical variable before inclusion in the model with categories: 0 – 0.5 years, 0.5 – 1 year, 1 – 5 years, 5 – 9 years 9 – 12 years and 12 – 15 years. The reference age-group chosen was '5–9 years' as the number of participants in this group was large enough to allow better distinction of the associations in other groups.

The variables were tested in univariable analysis and were all included in the multivariable analysis. No backward or forward selection or minimisation of models was carried out. A likelihood-ratio chi-square was used to test whether interaction terms incorporated into the models were statistically significant. In order to account for repeated sampling of individuals, I calculated robust standard errors with clustering allowance. Where observations were missing data on the variables studied, they were excluded from the analyses. Multicollinearity of variables included in the model was assessed by calculating variance inflation factors (VIF), with a square root of VIF > 2 used as a threshold to indicate multicollinearity (**9.3 Appendix 3**). In order to assign statistical significance to measures of association derived from the models, a probability value (*p*) cut-off of 0.05 was used. R statistical analyses software was used in all the analyses via RStudio version 1.1.463[460].

3.5 Results

3.5.1 Demography

From data collected between 1998 and 2016 at cross-sectional surveys carried out in Ngerenya, Chonyi and Junju, a total of 19,580 observations from 2,703 children were obtained (**Figure 3.3**). For 3 participants, complete and accurate registration data was missing from the cohort registry, and they were dropped out of the analysis. Additionally, 2,817 observations were from participants aged >15 years and were also excluded from the analysis. A total of 16,760 observations from 2,223 study participants collected over 9,134 person-years of observation were then included in the analysis.

From the weekly surveillance, 624,699 observations were obtained for the follow-up period included in this analysis. However, 67,462 observations were from individuals aged 15 years and older; hence 557,237 observations were included in the analysis. A break-down of the sample selection process is provided in **Figure 3.4**. Provided in **Table 3.2** are the demographic characteristics of study participants participating in the cross-sectional surveys, while **Table 3.3** describes the characteristics of the study participants participating in the weekly follow-up visits. There were relatively similar numbers of observations from males and females in each cohort, and while the percentage of observations from study participants falling in each age-group differed,

the majority of the observations came from children aged between 1 – 9 years in each cohort.

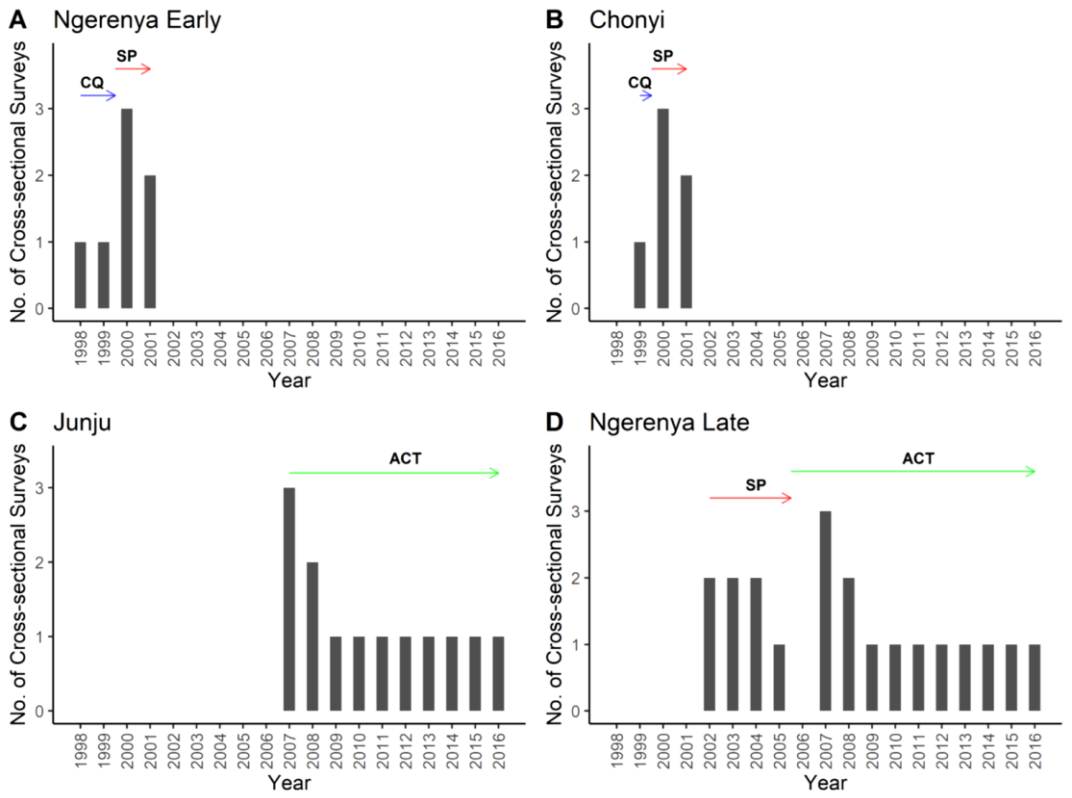


Figure 3.3: Summary of cross-sectional surveys carried out and malaria drug use per year for each cohort. Chloroquine (CQ) – blue lines; Sulphadoxine-Pyrimethamine (SP) – red lines; Artemisinin combination therapies (ACT) – green lines. Figure from Muthui *et al*. (2019)[454].

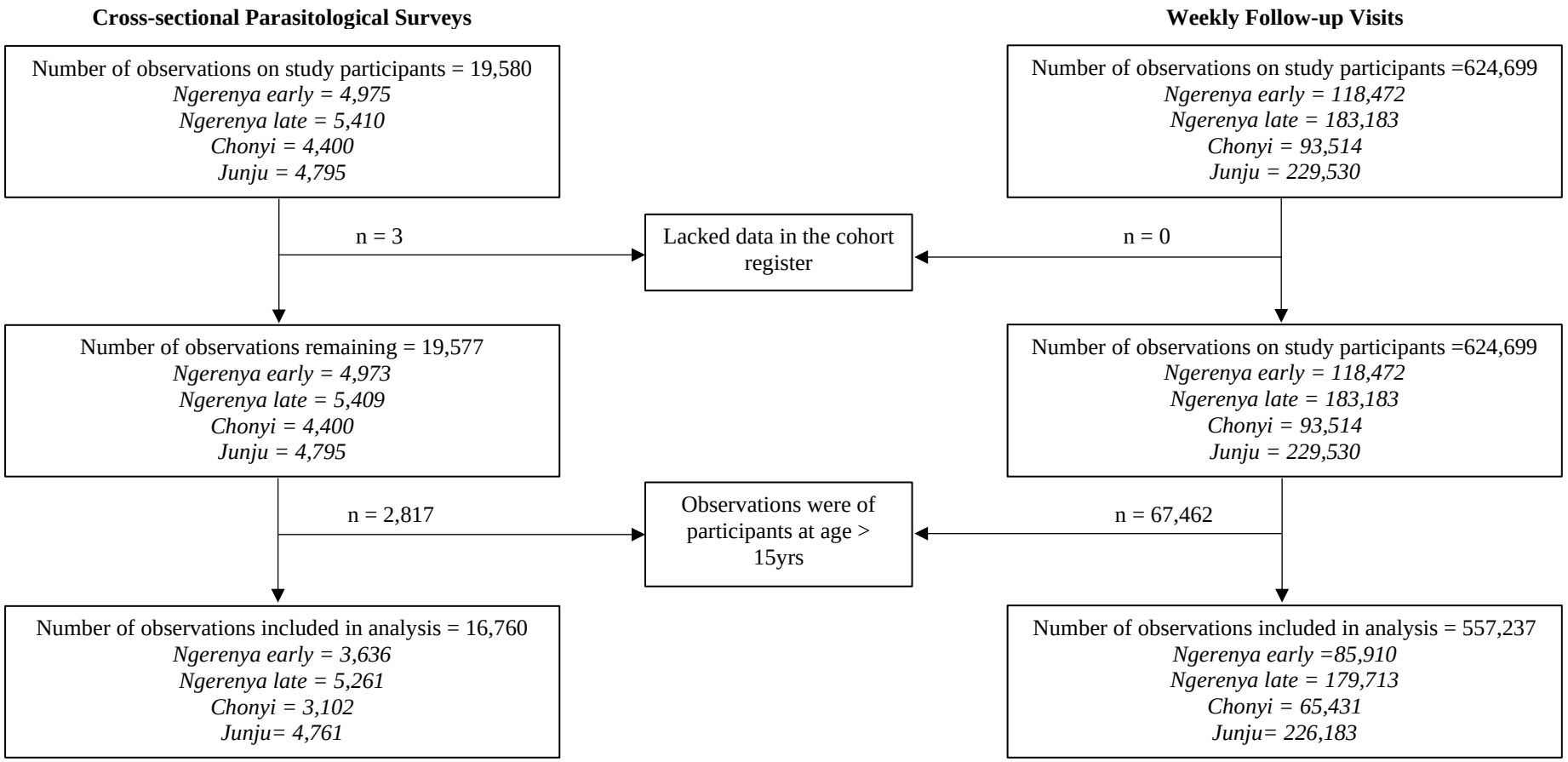


Figure 3.4: Flow diagram of the selection of observations from the Kilifi Malaria Longitudinal Cohort (KMLRC). Cross-sectional surveys and weekly follow-up visits were carried out on children recruited into the KMLRC. Reasons for exclusion are indicated at each step. Figure from Muthui *et al*. (2019)[454].

Table 3.2: Demographic characteristics of the children participating in the cross-sectional surveys

	Cohort			
	Ngerenya		Chonyi	Junju
	Early	Late		
Total number of observations from study participants	3636	5261	3102	4761
Number of observations from females (%)	1714 (47.1)	2417 (45.9)	1513 (48.8)	2391 (50.2)
Person-years of follow-up	882	4164	984	3104
Number per age group (%)				
<0.5 years	151 (4.2)	133 (2.5)	117 (3.8)	77 (1.6)
0.5 - 1 year	157 (4.3)	186 (3.5)	152 (4.9)	182 (3.8)
1 - 5 years	1199 (33.0)	1745 (33.2)	970 (31.2)	1577 (33.1)
5 -9 years	1078 (29.6)	1900 (36.1)	957 (30.9)	1792 (37.6)
9-12 years	725 (19.9)	889 (16.9)	598 (19.3)	729 (15.3)
12-15 years	326 (9.0)	408 (7.8)	308 (9.9)	404 (8.5)
Number of asexual parasite positive observations (%)	984 (27.1)	199 (3.8)	1183 (38.1)	850 (17.9)
Number of gametocyte positive observations (%)	164 (4.5)	20 (0.4)	142 (4.6)	38 (0.8)
Number of malaria episodes*	899	419	530	2941
Missing data (%)				
Gametocyte density	0	47(0.9)	0	71 (1.5)
Asexual parasite density	0	34(0.6)	0	69 (1.4)
Temperature	432 (11.9)	22 (0.4)	21 (0.7)	0

*Malaria episodes calculated from the weekly follow-up data for study participants with complete data on gametocyte density (summaries of the other data, except for gametocyte prevalence, include individuals with missing gametocyte data).

Table 3.3: Demographic characteristics of the children participating in the weekly follow-up visits

	Cohort			
	Ngerenya		Chonyi	Junju
	early	late		
Total number of observations from study participants	85910	179713	65431	226183
Number of observations from females (%)	41011 (47.7)	82995 (46.2)	31780 (48.6)	113122 (50.0)
Number per age group (%)				
<0.5 years	3612 (4.2)	5032 (2.8)	2714 (4.1)	4968 (2.2)
0.5 - 1 year	3818 (4.4)	6326 (3.5)	2937 (4.5)	7497 (3.3)
1 - 5 years	29211 (34.0)	57704 (32.1)	20434 (31.2)	76800 (34.0)
5 -9 years	25393 (29.6)	63612 (35.4)	20074 (30.7)	76496 (33.8)
9-12 years	16577 (19.3)	32156 (17.9)	12693 (19.4)	37452 (16.6)
12-15 years	7299 (8.5)	14883 (8.3)	6579 (10.1)	22970 (10.2)
Number of asexual parasite positive observations (%)	4114 (4.8)	1072 (0.6)	3015 (4.6)	5900 (2.6)
Number of gametocyte positive observations (%)	179 (0.2)	73 (0.04)	180 (0.3)	69 (0.03)
Number of malaria episodes	1055	349	605	3493

3.5.2 Parasite prevalence over time

I analysed the variation in the proportion of study participants with a parasite-positive blood film (asexual parasites or gametocytes) over the follow-up period included in this study (**Figure 3.2**). For all the three cohorts, gametocyte prevalence was much lower than asexual parasite prevalence. There was a general trend toward decreased parasite prevalence over time in all cohorts (Spearman's rank correlation $\rho = 0.78$, $p < 0.0001$). An analysis by cohort, also indicated a decrease in parasite prevalence in Ngerenya Late ($\rho = 0.8$, $p = 0.0003$) however, the temporal variation was random in Junju ($\rho = -0.04$, $p = 0.9$). An analysis in Ngerenya early and Chonyi cohorts is hampered as there are few data points to allow for a robust analysis.

3.5.3 Parasite density over time

In Ngerenya early, asexual parasite densities did not significantly differ over time ($\rho = -0.05$, $p = 0.09$, **Figure 3.5**). The relatively constant asexual parasite density was also mirrored in Ngerenya late ($\rho = -0.02$, $p = 0.79$), though from 2005 far fewer cases where detected owing to the decline in malaria transmission in the area. In Junju, asexual parasite densities declined marginally over time ($\rho = -0.09$, $p = 0.006$). Similarly, there was a slight decline in asexual parasite densities in Chonyi ($\rho = -0.10$, $p = 0.001$), but overall, the parasite density remained high throughout follow-up. No variation in gametocyte densities over time was observed in all the cohorts (**Figure 3.6**). However, the number of individuals contributing to this was small, thus making it difficult to draw a definite conclusion.

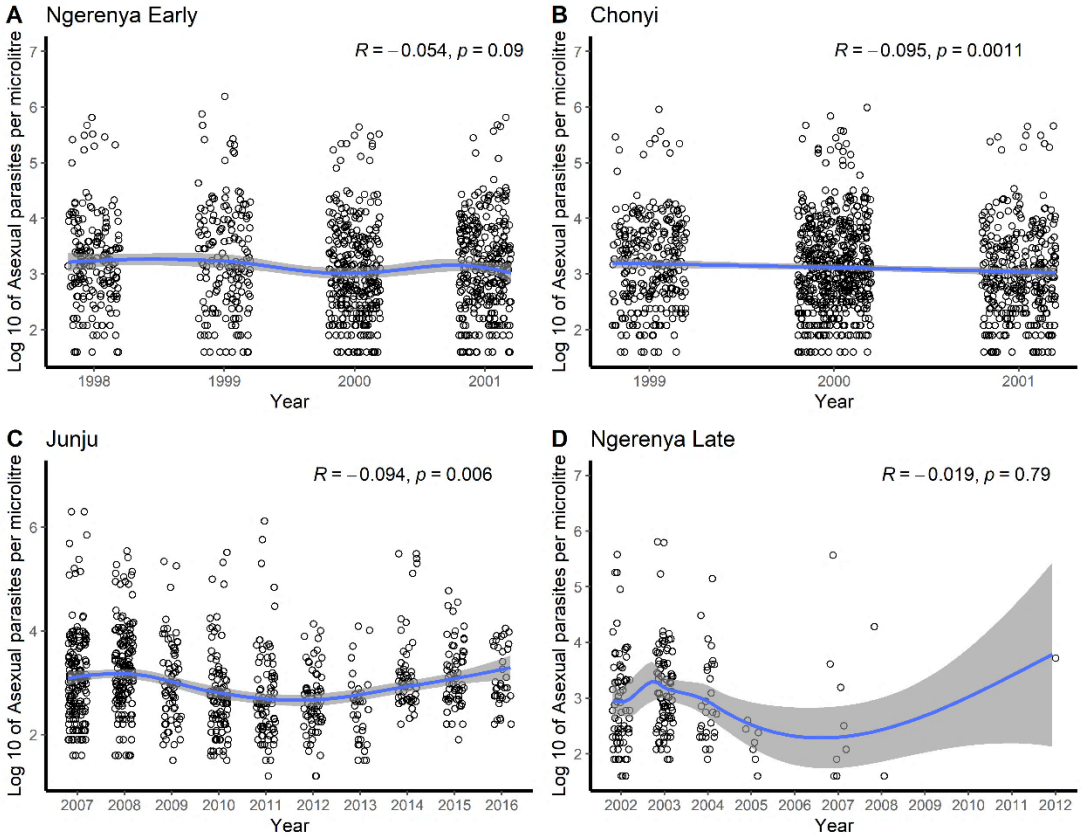


Figure 3.5: Variation in asexual parasite densities in Ngerenya, Chonyi and Junju cohorts over time. Scatter plots showing the temporal fluctuation of asexual parasite densities for each of the cohorts. (A) Ngerenya early, (B) Chonyi, (C) Junju and (D) Ngerenya late. Regression analysis (Locally weighted Scatterplot Smoothing (LOESS) smoothing) was used to generate the data (means – blue line; and confidence intervals – shaded grey area) used to plot the smooth line through the scatterplot to analyse the trend in density over time. Spearman's rank correlation coefficients and associated *p* – values are also shown on the graph.

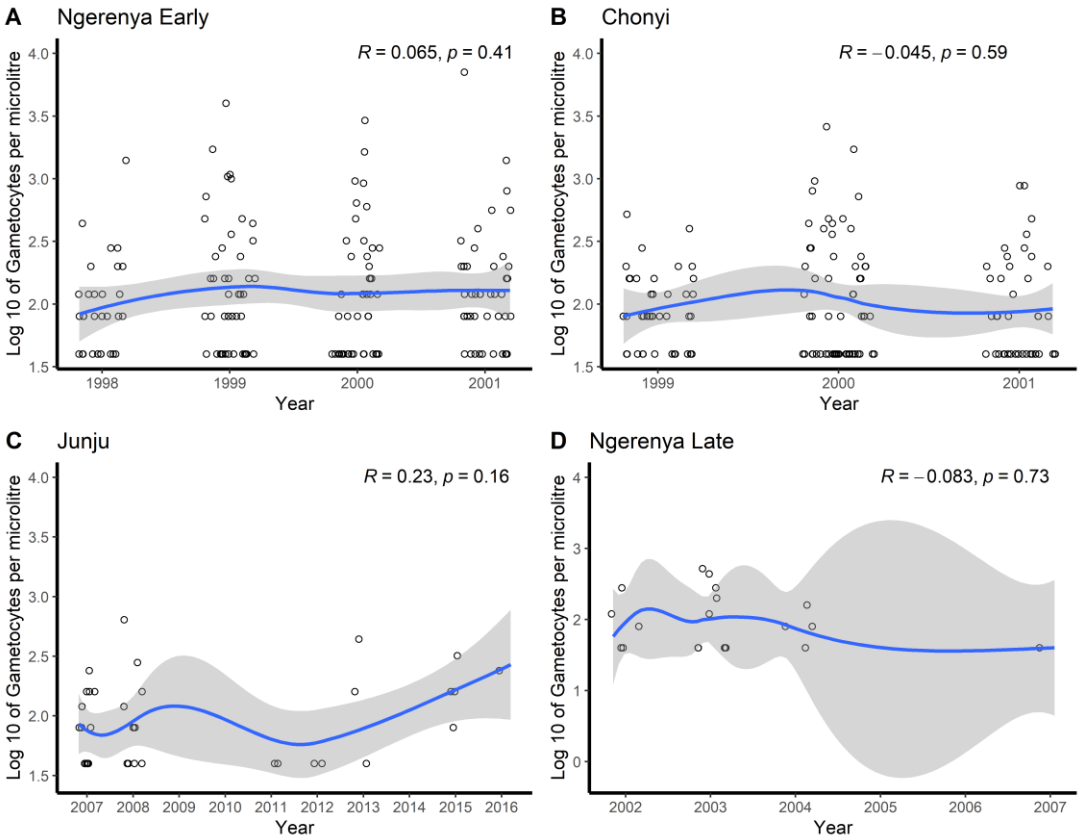


Figure 3.6: Variation in gametocyte densities in Ngerenya, Chonyi and Junju cohorts over time. Scatter plots showing the temporal fluctuation of gametocyte densities over time. (A) Ngerenya early, (B) Chonyi, (C) Junju and (D) Ngerenya late. For Ngerenya late, the x-axis is truncated after 2007 as there were no more microscopically detected gametocytes recorded. Regression analysis (Locally weighted Scatterplot Smoothing (LOESS) smoothing) was used to generate the data (means – blue line; and confidence intervals – shaded grey area) used to plot the smooth line through the scatterplot to analyse the trend in density over time. Spearman's rank **c**orrelation coefficients and associated *p* – values are also shown on the graph.

3.5.4 Age and parasite prevalence

I further examined the effect of age on the proportion of parasite-positive observations (**Figure 3.7**). In all four cohorts, asexual parasitaemia increased with age until about nine years of age ($p<0.05$, pairwise comparison of proportions with Bonferroni correction for multiple testing). No significant differences in parasite prevalence were observed among the 5 – 9-year, 9 – 12-year and 12 – 15-year age groups for all cohorts but Ngerenya late. For Ngerenya late there was a significant decline in asexual parasite prevalence in the older age groups compared to the 5 – 9-year age group (5 -9-year vs. 9 – 12-year, $p<0.0001$ and 9 – 12-year vs 12 – 15year, $p<0.0001$). The proportion with gametocytaemia was much lower than that of asexual parasitaemia (particularly

in Junju cohort). There were no statistically significant differences in the gametocyte prevalence between the age groups for Chonyi, Junju and Ngerenya late. For Ngerenya early, gametocyte prevalence was higher in the 1 – 5-year age groups in comparison to the 9 – 12-year ($p = 0.0002$) and 12 – 15-year ($p = 0.005$) age groups.

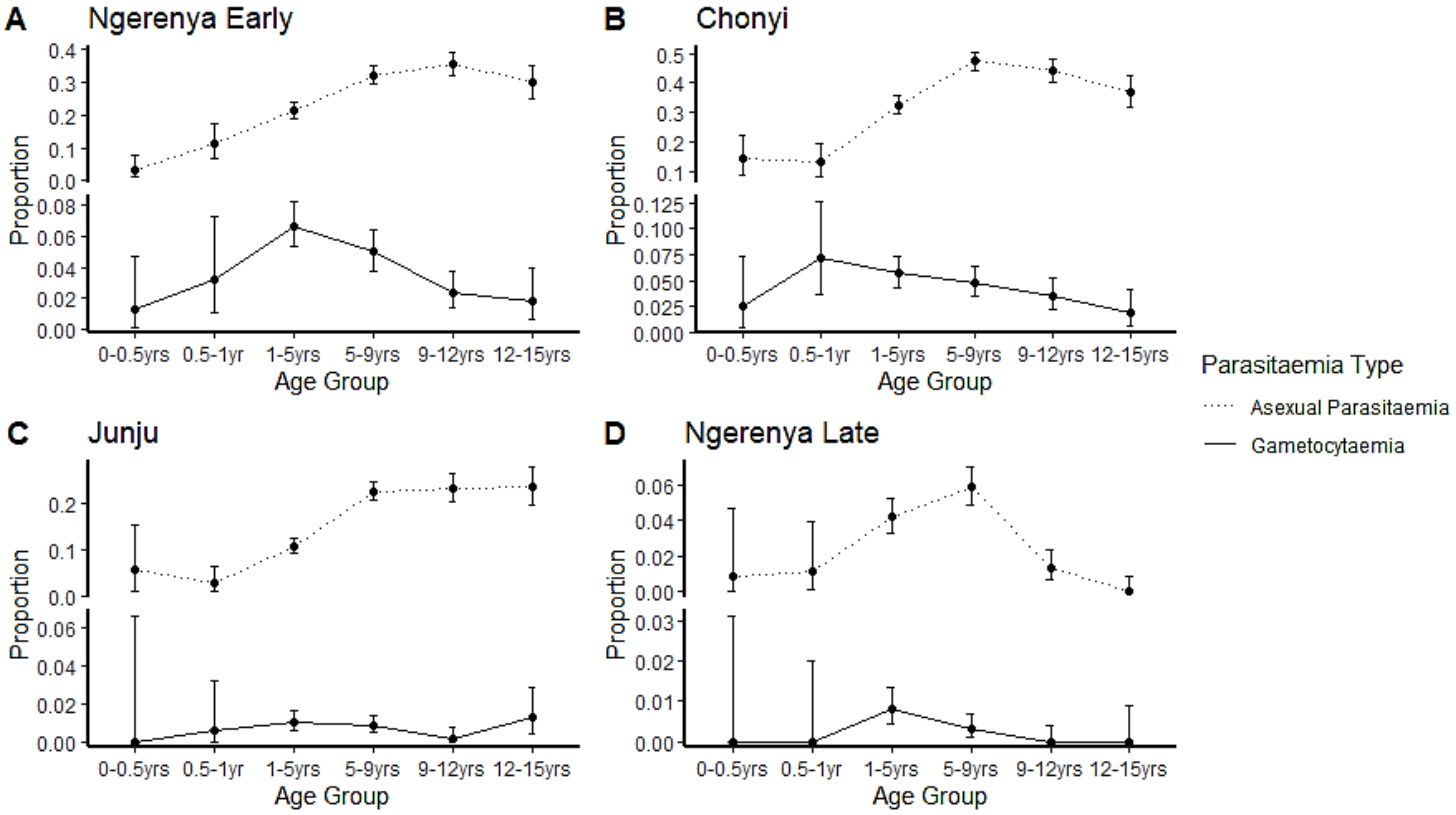


Figure 3.7: Parasite prevalence by age. Line graphs showing the variation prevalence of asexual parasitaemia and gametocytaemia in the different age groups (0 – 0.5 years, 0.5 – 1 year, 1 – 5 years, 5 – 9 years, 9 – 12 years and 12 – 15 years) in (A) Ngerenya early, (B) Chonyi, (C) Junju and (D) Ngerenya late. The error bars indicate 95% confidence intervals. Figure from Muthui *et al*. (2019)[454].

3.5.5 Distribution of parasite prevalence

I analysed the distribution of the number of parasite positive events per study participant in each of the cohorts. First, the number of blood films taken per participant was determined, then the most frequent 'number of blood films per participant' was determined for each cohort. The analysis was then limited to individuals who had had the same number of blood films taken (determined separately for each cohort) to avoid potential bias. I then fitted predicted frequencies for a binomial distribution over the distribution of asexual parasite and gametocyte positive events. What was evident was that the distribution of asexual parasite positive events did not appear to follow a binomial distribution (**Figure 3.8**), while that of gametocyte positive events was approximately binomial (**Figure 3.9**). The distribution of asexual parasite positive was likely due to a higher than predicted number of individuals presenting with multiple asexual parasite positive events.

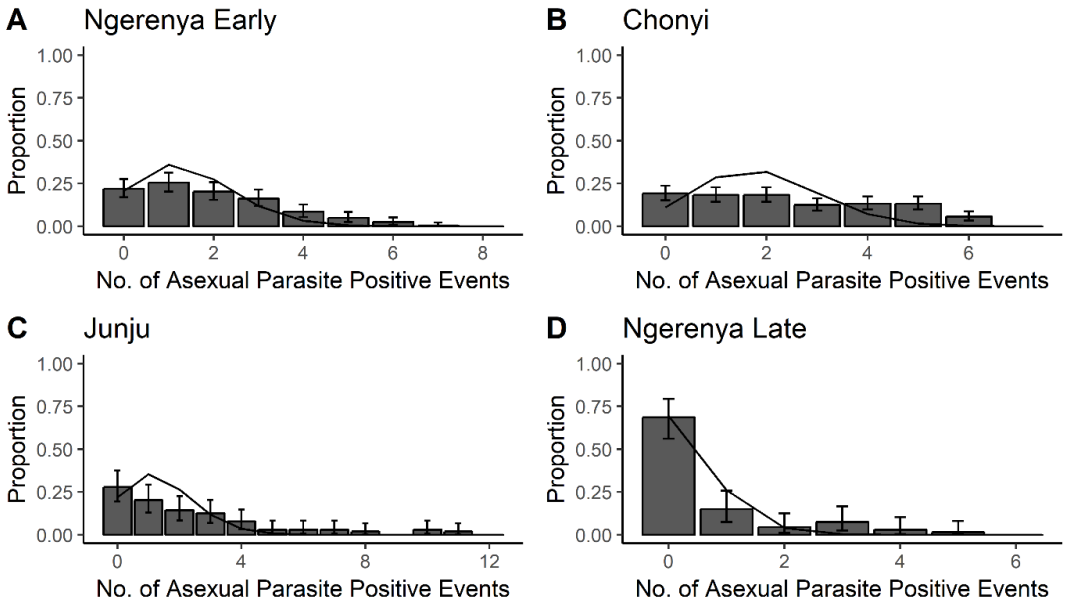


Figure 3.8: Distribution of individuals with multiple instances of asexual parasitaemia. Bar plots showing the proportion of individuals positive for asexual parasites. This was restricted to individuals who had the same number of blood films taken, and the blood film number with the highest frequency. (A) Ngerenya early, (B) Chonyi, (C) Junju and (D) Ngerenya late. The solid line indicates expected values for a binomial distribution. Figure from Muthui *et al*. (2019)[454].

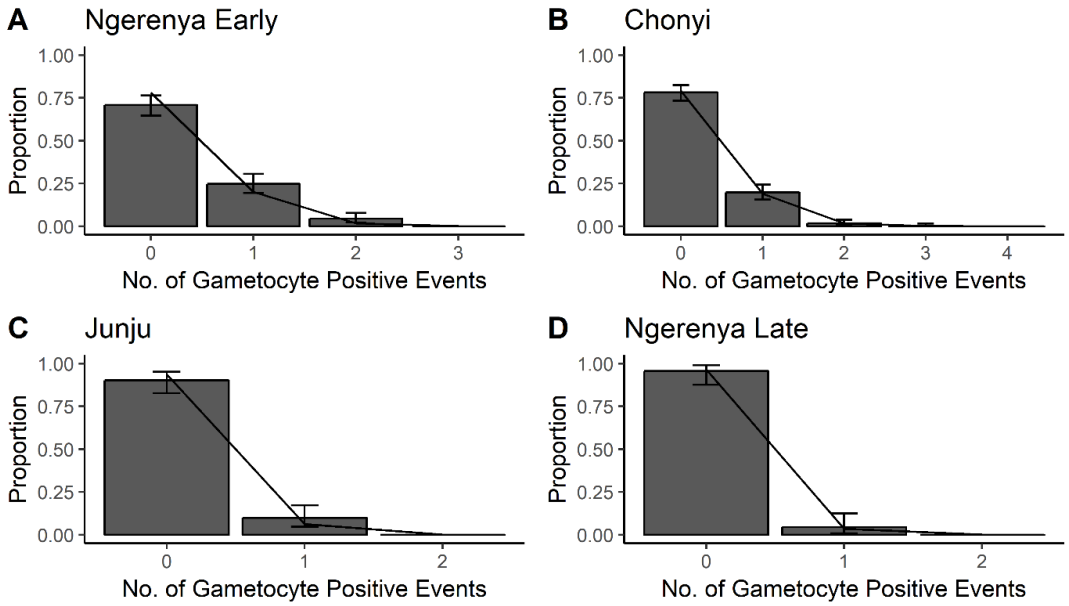


Figure 3.9: Distribution of individuals with multiple instances of gametocytaemia. Bar plots showing the proportion of individuals positive for gametocytes**.** This was restricted to individuals who had the same number of blood films taken, and the blood film number with the highest frequency. (A) Ngerenya early, (B) Chonyi, (C) Junju and (D) Ngerenya late. The solid line indicates expected values for a binomial distribution. Figure from Muthui *et al*. (2019)[454].

3.5.6 Factors predicting gametocyte positivity

3.5.6.1 Overall analysis

To understand the association between the various variables and gametocyte positivity, I fitted models to predict gametocyte positivity. The covariates used were asexual parasite density, age, year, number of malaria episodes and whether an individual was gametocyte positive, asexual parasite positive, or had a malaria episode during the previous survey. Two count models, Poisson and logistic regression models were evaluated and, based on the lower AIC value, the logistic regression model was considered a better fit for the data. I also compared models that included asexual parasitaemia as a binary variable (positive (1) versus negative (0)) and as a continuous variable and found that using asexual parasitaemia as a binary variable better fit the data.

From the univariable analysis, asexual parasite positivity, number of malaria episodes and being gametocyte or asexual parasite positive during the previous year were associated with increased odds of being gametocyte positive, and this was statistically significant ($p < 0.05$) (**Table 3.4**). Relative to Chonyi (a high transmission setting),

residing in Junju (moderate transmission setting) or Ngerenya late (low transmission setting) was associated with lower odds of being gametocyte positive. With age, being between 0 – 5 years of age was associated with increased odds of gametocyte positivity relative to the 5 – 9-year age group. However, being older (9 – 15 age groups) was associated with decreased odds of being gametocyte positive. These associations were statistically significant for all but the 0 – 0.5-year age group.

In the multivariable analysis, asexual parasite positivity, age, transmission setting and being gametocyte positive in the prior survey remained strong independent predictors of gametocyte positivity. Being asexual parasite positive was associated with an approximately five-fold increase in odds of gametocyte positivity (95% CI: 3. 34 – 6. 22, p <0.0001). For age, being between 0.5 – 1 year as well as 1 – 5 years was associated with increased odds of gametocyte positivity (2. 21-fold (95% CI: 1.21 – 4. 04, p = 0. 01) and 1. 7-fold (95% CI: 1. 26 – 2. 29, p = 0.0005) respectively). Relative to Chonyi, residing in Junju was associated with 76% decreased odds of gametocyte positivity (95% CI: 84% – 63%, p <0.0001) while residing in Ngerenya during the period of low transmission was associated with 80% decreased odds of gametocyte positivity (95% CI: 89% – 66%, p <0.0001). Having a malaria episode was associated with a 1. 21-fold increased odds of gametocyte positivity (95% CI: 1.13 – 1. 29-fold, p <0.0001) while being gametocyte positive in the prior survey associated with a 2-fold increase in odds of gametocyte positivity (95% CI: 1.22, 3. 18-fold, p = 0.005). Being asexual parasite positive at the previous survey was no longer predictive in the multivariable analysis and hence may only impact gametocytaemia through its relationship with repeated parasite positivity.

Table 3.4: Logistic regression model predicting gametocyte positivity

	Univariable Analysis			Multivariable Analysis		
Covariate	**Odds ratio**	**95% CI**	***p value***	**Odds ratio**	**95% CI**	***p value***
Asexual parasite positive	6.70	5.38, 8.34	**<0.0001**	4.56	3.34, 6.22	<0.0001
Age group						
5 - 9 years	1.00	.	.	1.00	.	.
0 - 0.5 years	0.53	0.22, 1.31	0.1683	1.62	0.37, 7.01	0.5197
0.5 - 1 year	1.21	0.71, 2.08	0.4842	2.21	1.21, 4.04	0.0100
1 - 5 years	1.44	1.12, 1.85	**0.0041**	1.70	1.26, 2.29	0.0005
9 -12 years	0.62	0.43, 0.90	**0.0293**	0.63	0.42, 0.95	0.0295
12 - 15 years	0.55	0.32, 0.94	**0.0118**	0.69	0.38, 1.26	0.2251
Cohort						
Chonyi	1.00	.	.	1.00	.	.
Junju	0.17	0.12, 0.25	**<0.0001**	0.24	0.16, 0.37	<0.0001
Ngerenya early	0.98	0.77, 1.25	0.8991	1.16	0.87, 1.54	0.3210
Ngerenya late	0.08	0.05, 0.13	**<0.0001**	0.20	0.11, 0.34	<0.0001
No. of malaria episodes[i]	1.31	1.23, 1.38	<0.0001	1.21	1.13, 1.29	<0.0001
Malaria episodes in the prior survey	1.04	0.89, 1.21	0.6268	0.89	0.71, 1.11	0.2974
Gametocyte positive in the prior survey	4.58	2.95, 7.13	**<0.0001**	1.97	1.22, 3.18	0.0053
Asexual parasite positive in the prior survey	2.16	1.68, 2.80	**<0.0001**	0.86	0.64, 1.15	0.3171

[i] Number of malaria episodes – the sum of the number of malaria episodes occurring in the period leading up to a cross-sectional survey (corrected for the interval between days).
P values in bold are statistically significant (<0.05).
CI – confidence interval.

I then also explored the possible predictors of gametocyte positivity in a subset of individuals who were positive for asexual parasites (**Table 3.5**). A summary of study participants positive for either asexual parasites or gametocytes or both is presented in **9.3 Appendix 3**. Unlike in the overall analysis (**Table 3.4**), previous survey positivity for asexual parasites was associated with a decreased odds of gametocyte positivity in the univariable analysis. While this could relate to individuals with repeated parasite positivity having better developed natural immunity to malaria, the association is no longer evident in the multivariable analysis. Again, previous survey positivity may not independently predict gametocyte carriage.

Like the overall analysis, participants under the age of five, having a malaria episode as well as being gametocyte positive in the prior survey, were associated with increased odds of gametocyte positivity. For cohort, residing in Junju was associated with reduced odds of being gametocyte positive; however, Ngerenya late cohort was no longer associated with reduced odds of gametocytaemia. This could indicate that factors unrelated to asexual parasite prevalence influenced gametocyte positivity in Junju.

Table 3.5: Logistic regression model predicting gametocyte positivity in individuals positive for asexual parasites

Covariate	Univariable analysis			Multivariable analysis		
	Odds ratio	**95% CI**	***p* value**	**Odds ratio**	**95% CI**	***p* value**
Age group						
5 - 9 years	1.00	.	.	1.00	.	.
0 - 0.5 years	2.26	0.66, 7.76	0.1961	N/A	N/A	N/A
0.5 - 1 year	5.60	2.80, 11.20	**<0.0001**	6.54	2.76, 15.51	<0.0001
1 - 5 years	2.34	1.68, 3.26	**<0.0001**	2.43	1.65, 3.57	<0.0001
9 -12 years	0.77	0.50, 1.18	0.2312	0.84	0.5, 1.42	0.5103
12 - 15 years	0.34	0.15, 0.80	**0.0131**	0.45	0.17, 1.16	0.0969
Cohort						
Chonyi	1.00	.	.	1.00	.	.
Junju	0.46	0.30, 0.71	**0.0004**	0.50	0.31, 0.81	0.0044
Ngerenya early	1.16	0.84, 1.60	0.3559	1.17	0.79, 1.74	0.4413
Ngerenya late	0.78	0.40, 1.53	0.4726	0.77	0.4, 1.51	0.4552
Number of malaria episodes[i]	1.17	1.08, 1.27	0.0001	1.10	1.01, 1.21	0.0246
Number of malaria episodes in the prior survey	0.99	0.77, 1.26	0.9086	0.82	0.6, 1.1	0.1843
Gametocyte positive in the prior survey	2.98	1.73, 5.15	**<0.0001**	2.28	1.27, 4.1	0.0058
Asexual parasite positive in the prior survey	0.69	0.49, 0.96	**0.0284**	0.72	0.51, 1.02	0.0671

[i] Number of malaria episodes – the sum of the number of malaria episodes occurring in the period leading up to a cross-sectional survey (corrected for the interval between days).
P values in bold are statistically significant (<0.05).
CI – confidence interval.
N/A – sample size insufficient for analysis.

3.5.6.2 Impact of malaria episodes on gametocyte carriage

The interaction between malaria episodes and cohort was assessed to understand the relationship between the two variables (**Table 3.6**). Associations similar to the main analysis (**Table 3.4**) were observed. Asexual parasite positivity, participants under the age of five, having a malaria episode as well as being gametocyte positive in the prior survey, were associated with increased odds of gametocyte positivity.

A likelihood-ratio chi-square test indicated a significant interaction between cohort and malaria episodes (p = 0.007). For the univariable analysis, in both Junju and Ngerenya late there appeared to be a significant variation in the association between the number of malaria episodes and the odds of gametocytaemia (Junju: 43% decreased odds (95% CI: 63% – 11%, p = 0.01) and Ngerenya late: 1.8-fold increased odds (95% CI: 1.35 – 2.33, p<0.0001). However, after adjusting for the other variables in the multivariable analysis, no strong associations were seen between the cohorts and the number of malaria episodes. Additionally, as there seemed to be a stronger association between asexual parasite positivity and increased odds of gametocyte carriage in Junju and Ngerenya late, I tested the interaction between asexual parasitaemia and cohort. A significant interaction between cohort and asexual parasite positivity was also observed (p <0.0001). Relative to Chonyi, there was an observed increased odds of gametocyte positivity with asexual parasite positivity only in Junju (p = 0.0005) and Ngerenya late (p = 0.001).

Table 3.6: Logistic regression model predicting gametocyte positivity with interaction analysis

Covariate	Univariable analysis			Multivariable analysis		
	Odds ratio	**95% CI**	***p value***	**Odds ratio**	**95% CI**	***p value***
Asexual parasite positive	6.70	5.38, 8.34	**<0.0001**	3.16	2.03, 4.91	<0.0001
Age group						
5–9 years	1.00	.	.	1.00	.	.
0–0.5 years	0.53	0.22, 1.31	0.1683	1.51	0.36, 6.31	0.568
0.5–1 year	1.21	0.71, 2.08	0.4842	2.12	1.16, 3.86	0.0142
1–5 years	1.44	1.12, 1.85	**0.0041**	1.69	1.25, 2.28	0.0006
9–12 years	0.62	0.43, 0.90	**0.0293**	0.67	0.44, 1.01	0.0584
12–15 years	0.55	0.32, 0.94	**0.0118**	0.71	0.39, 1.3	0.2701
Cohort						
Chonyi	1.00	.	.	1.00	.	.
Junju	0.17	0.12, 0.25	**<0.0001**	0.11	0.05, 0.26	<0.0001
Ngerenya early	0.98	0.77, 1.25	0.8991	1	0.66, 1.53	0.9871
Ngerenya late	0.08	0.05, 0.13	**<0.0001**	0.07	0.03, 0.13	<0.0001
Number of malaria episodes[i]	1.31	1.23, 1.38	<0.0001	1.2	1.07, 1.36	0.0027
Number of malaria episodes in the prior survey	1.04	0.89, 1.21	0.6268	0.91	0.72, 1.14	0.3964
Gametocyte positive in the prior survey	4.58	2.95, 7.13	<0.0001	1.94	1.21, 3.1	0.0056
Asexual parasite positive in the prior survey	2.16	1.68, 2.80	**<0.0001**	0.82	0.62, 1.09	0.1673
Asexual parasite positive: Chonyi	1.00	.	.	1.00	.	.
Asexual parasite positive: Junju	7.02	2.84, 17.37	**<0.0001**	5.25	2.05, 13.45	0.0006
Asexual parasite positive: Ngerenya early	1.07	0.66, 1.73	0.3557	0.99	0.56, 1.75	0.9728
Asexual parasite positive: Ngerenya late	13.66	5.00, 37.31	**<0.0001**	8.74	2.34, 32.68	0.0013
Chonyi: Number of malaria episodes	1.00	.	.	1.00	.	.
Junju: Number of malaria episodes	0.57	0.37, 0.89	0.0123	0.67	0.45, 1.02	0.059

Covariate	Univariable analysis			Multivariable analysis		
	Odds ratio	**95% CI**	***p value***	**Odds ratio**	**95% CI**	***p value***
Ngerenya early: Number of malaria episodes	1.01	0.89, 1.15	0.8206	1.09	0.94, 1.27	0.2515
Ngerenya late: Number of malaria episodes	1.77	1.35, 2.33	<0.0001	1.35	0.8, 2.29	0.2591

[i] The sum of the number of malaria episodes occurring in the period leading up to a cross-sectional survey (corrected for the interval between days).
P values in bold are statistically significant (<0.05).
CI – confidence interval.

Noting the possibility of an interaction between cohort and malaria episodes, and the difference in follow-up period among the cohorts, the impact of drug regimen on gametocyte positivity was also investigated. For this, the dataset was divided into two time-periods based on ACT introduction: before 2006 and after 2006. Post-ACT introduction, there was a sharp drop in gametocyte prevalence, dropping from approximately 4% to 0.5% (**Figure 3.10**). The impact of the drug regimen was further tested in logistic models, considering only malaria episodes that only occurred within 28 days prior to a cross-sectional survey.

In the univariable analysis, associations between the different age groups and gametocyte carriage differed from the overall analysis in **Table 3.4**. However, after adjusting for the other variables in the multivariable analysis, the associations remained similar to the overall analysis with increased gametocyte carriage in children under five years of age. Additionally, similar associations as in the overall analysis (**Table 3.4**) were seen between asexual parasite positivity, malaria episodes and gametocyte positivity in the previous survey. The analysis also showed that before ACT introduction, before 2006 (**Table 3. 7**), the number of malaria episodes were associated with an increased risk of gametocyte positivity (OR 1. 15, 95% CI: 1. 05 – 1. 25, p = 0. 003) while recent malaria episodes (episodes occurring within 28 days of a cross-sectional survey) were associated with an approximately threefold increased risk of gametocyte positivity (95% CI: 1. 5 – 4.52, p = 0.0007).

In the post-2006 analysis (**Table 3.8**), only asexual parasite positivity remained associated with gametocyte positivity. Furthermore, the number of malaria episodes a participant had, and recent malaria episodes, were no longer predictors of gametocyte positivity (OR 0.71, 95% CI: 0.44 – 1.15, p = 0.17 and OR 2.1, 95% CI: 0.19 – 22.64, p = 0.54, respectively). The post-ACT period includes only Junju and Ngerenya late cohorts where gametocyte prevalence is markedly low, and this is reflected in the lack of associations with the other key predictors identified in previous analysis, i.e. age and gametocyte positivity in the previous survey.

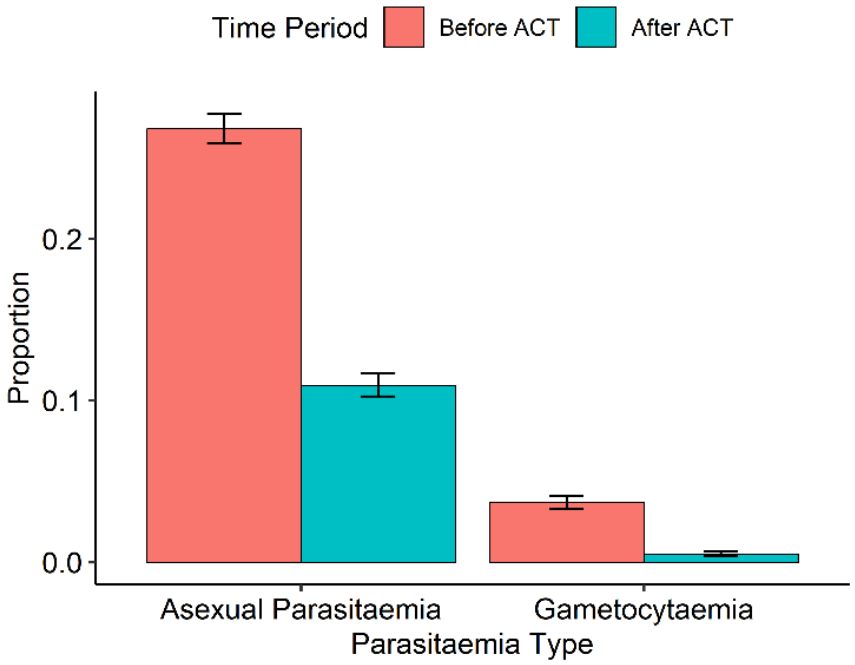


Figure 3.10: Parasite prevalence in the periods before and after ACT introduction. Bar plots showing the proportion of study participants positive for gametocytes or asexual parasites before and after the introduction of ACTs. Before ACTs and after ACTs, the prevalence of gametocytaemia was 4% and 0.5%, while the prevalence of asexual parasitaemia was 27% and 11% respectively. Figure from Muthui *et al*. (2019)[454].

Table 3.7: Logistic regression model predicting gametocyte positivity before ACT introduction

Covariate	Univariable analysis			Multivariable analysis		
	Odds ratio	**95% CI**	***p* value**	**Odds ratio**	**95% CI**	***p* value**
Asexual parasite positive	3.93	3.12, 4.95	**<0.0001**	3.5	2.56, 4.78	<0.0001
Age group						
5–9 years	1.00	.	.	1.00	.	.
0–0.5 years	0.37	0.15, 0.91	**0.0310**	1.55	0.37, 6.44	0.5456
0.5–1 year	1.06	0.60, 1.87	08350	1.92	1.04, 3.55	0.0363
1–5 years	1.31	1.00, 1.70	**0.0488**	1.64	1.19, 2.25	0.0022
9–12 years	0.72	0.50, 1.06	0.0937	0.74	0.48, 1.14	0.1715
12–15 years	0.50	0.26, 0.96	**0.0359**	0.57	0.28, 1.19	0.1345
Cohort						
Chonyi	1.00	.	.	1.00	.	.
Ngerenya early	0.98	0.77, 1.25	0.8991	1.1	0.82, 1.46	0.5276
Ngerenya late	0.20	0.12, 0.32	**<0.0001**	0.34	0.2, 0.57	0.0001
Number of malaria episodes[i]	1.32	1.25, 1.40	<0.0001	1.15	1.05, 1.25	0.0032
Number of malaria episodes in the prior survey	1.18	0.95, 1.47	0.1353	0.86	0.67, 1.11	0.2462
Gametocyte positive in the prior survey	2.76	1.75, 4.34	**<0.0001**	2.03	1.24, 3.32	0.0046
Asexual parasite positive in the prior survey	1.40	1.07, 1.83	**0.0146**	0.87	0.64, 1.18	0.3690
Recent epiode (within 28 days)						
No	1.00	.	.	1.00	.	.
Yes	5.60	4.03, 7.79	**<0.0001**	2.6	1.5, 4.52	0.0007

[i] The sum of the number of malaria episodes occurring in the period leading up to a cross-sectional survey (corrected for the interval between days).
P values in bold are statistically significant (<0.05).
CI – confidence interval.

Table 3.8: Logistic regression model predicting gametocyte positivity after ACT introduction

Covariate	Univariable analysis			Multivariable analysis		
	Odds ratio	**95% CI**	***p value***	**Odds ratio**	**95% CI**	***p value***
Asexual parasite positive	38.60	16.77, 88.81	**<0.0001**	23.96	7.5, 76.59	<0.0001
Age group						
5–9 years	1.00	.	.	1.00	.	.
0–0.5 years	N/A	N/A	N/A	N/A	N/A	N/A
0.5–1 year	0.74	0.10, 5.56	0.7689	6.41	0.91, 45.04	0.0617
1–5 years	1.37	0.66, 2.89	0.3944	2.19	0.91, 5.26	0.0795
9–12 years	0.12	0.02, 0.94	**0.0435**	0.18	0.02, 1.4	0.1008
12-15 years	1.18	0.42, 3.26	0.7545	1.61	0.53, 4.87	0.3959
Cohort						
Junju	1.00	.	.	1.00	.	.
Ngerenya late	0.04	0.01, 0.28	**0.0013**	0.16	0.02, 1.66	0.1251
Number of malaria episodes[i]	0.93	0.66, 1.30	0.6536	0.71	0.44, 1.15	0.1653
Number of malaria episodes in the prior survey	1.16	0.83, 1.62	0.3815	1.07	0.69, 1.68	0.7513
Gametocyte positive in the prior survey	7.58	1.01, 56.90	**0.0489**	1.84	0.26, 12.87	0.5366
Asexual parasite positive in the prior survey	3.44	1.58, 7.49	**0.0019**	0.64	0.27, 1.54	0.3235
Recent episode (within 28 days)						
No	1.00	.	.	1.00	.	.
Yes	1.01	0.14, 7.43	0.994	2.1	0.19, 22.64	0.5418

[i] The sum of the number of malaria episodes occurring in the period leading up to a cross-sectional survey (corrected for the interval between days).
P values in bold are statistically significant (<0.05).
CI – confidence interval.
N/A - sample size insufficient for analysis

3.5.6.3 Effect of human genetic factors on gametocyte carriage

Finally, the impact of host genetic factors on gametocyte positivity was evaluated in a subset of asexual parasite positive individuals for whom genotype data was available (**9.3 Appendix 3**). Genetic factors tested were sickle cell status, α-thalassaemia status and blood group. Only nine children had sickle cell disease; hence odds ratios are not presented. In the analysis, associations between age, cohort, asexual parasite positivity in the previous survey and gametocyte positivity in the previous survey were similar to associations seen in the analysis limited to the asexual parasite positive individuals only (**Table 3.5**). However, while malaria episodes were similarly associated with increased odds of gametocyte positivity in the univariable analysis, no association was seen in the multivariable analysis (**Table 3.9**). Subsetting the dataset likely diminished association between malaria episodes and gametocyte carriage. Sickle cell trait did not appear associated with gametocyte positivity (OR 1.26, 95% CI: 0.73 – 2.2, p = 0. 41). Moreover, neither homozygosity (OR 0.60 95% CI: 0.28 – 1.28, p = 0.18) nor heterozygosity (OR 1.03, 95% CI: 0.68 – 1.57, p = 0.88) for α-thalassaemia appeared associated with gametocyte positivity.

Analysis was also carried out to evaluate the influence of blood group on gametocyte carriage (**Table 3.10**). This analysis was limited to fewer participants and excluded Chonyi cohort. Despite a further reduction in sample size, univariable and multivariable associations with age and asexual parasite positivity in the previous survey were similar to associations seen in the models limited to individuals typed for sickle and α-thalassaemia. However, gametocyte positivity in the previous survey was no longer associated with increased odds of gametocyte carriage in the multivariable analysis. Relative to Junju cohort, in both the univariable and multivariable analysis, Ngerenya early was associated with increased odds of gametocyte carriage while no association was seen for Ngerenya late cohort. There was no association between blood groups B and O, relative to the AB and A blood groups, with gametocyte positivity (OR 1.38, 95% CI 0.56 – 3.42, p = 0.49 and OR 0.93, 95% CI 0.45 – 1.92, p = 0.84, respectively). Similarly, no associations between the different genotypes and gametocytaemia were observed when sickle cell genotype, α-thalassaemia genotype and blood group were tested in the same model (**9.3 Appendix 3**).

Table 3.9: Logistic regression model predicting gametocyte positivity including sickle cell and α-thalassaemia genotype data

Covariate	Univariable analysis			Multivariable analysis		
	Odds ratio	**95% CI**	***p value***	**Odds ratio**	**95% CI**	***p value***
Age group						
5 - 9 years	1.00	.	.	1.00	.	.
0 - 0.5 years	1.6	0.20, 12.97	0.6565	N/A	N/A	N/A
0.5 - 1 year	4.08	1.68, 9.88	0.0018	4.49	1.33, 15.08	0.0152
1 - 5 years	2.42	1.62, 3.61	<0.0001	2.32	1.47, 3.66	0.0003
9 -12 years	0.64	0.35, 1.15	0.1332	0.59	0.29, 1.19	0.1392
12 - 15 years	0.34	0.12, 0.97	0.0433	0.35	0.1, 1.25	0.1059
Cohort						
Chonyi	1.00	.	.	1.00	.	.
Junju	0.45	0.27, 0.77	0.0032	0.44	0.24, 0.8	0.0074
Ngerenya early	1.32	0.84, 2.07	0.2323	1.17	0.67, 2.05	0.5839
Ngerenya late	0.79	0.38, 1.63	0.5175	0.63	0.29, 1.36	0.2406
No. of malaria episodes[i]	1.19	1.06, 1.33	0.0333	0.99	0.78, 1.25	0.9158
Number of malaria episodes in the prior survey	0.89	0.64, 1.23	0.4634	0.76	0.51, 1.12	0.1610
Gametocyte positive in the prior survey	2.95	1.52, 7.73	0.0014	2.24	1.08, 4.65	0.0309
Asexual parasite positive in the prior survey	0.65	0.43, 0.98	0.0415	0.69	0.44, 1.07	0.0940
Sickle cell genotype						
Normal	1.00	.	.	1.00	.	.
Heterozygous	0.95	0.57, 1.59	0.8554	1.26	0.73, 2.2	0.4076
Homozygous	N/A	N/A	N/A	N/A	N/A	N/A
α-thalassaemia genotype						
Normal	1.00	.	.	1.00	.	.
Heterozygous	1.06	0.73, 1.56	0.753	1.03	0.68, 1.57	0.8830

Covariate	Univariable analysis			Multivariable analysis		
	Odds ratio	**95% CI**	***p value***	**Odds ratio**	**95% CI**	***p value***
Homozygous	0.65	0.36, 1.19	0.1611	0.6	0.28, 1.28	0.1837

[i] The sum of the number of malaria episodes occurring in the period leading up to a cross-sectional survey (corrected for the interval between days).
P values in bold are statistically significant (<0.05).
CI – confidence interval.
N/A - sample size insufficient for analysis.

Table 3.10: Logistic regression model predicting gametocyte positivity including blood group data

Covariate	Univariable Analysis			Multivariable Analysis		
	Odds ratio	**95% CI**	***p value***	**Odds ratio**	**95% CI**	***p value***
Age group						
5–9 years	1.00	.	.	1.00	.	.
0–0.5 years	N/A	N/A	N/A	N/A	N/A	N/A
0.5–1 year	10.58	2.53, 44.19	0.0012	5.43	0.64, 45.96	0.1198
1–5 years	4.42	1.98, 9.84	0.0003	2.77	1.11, 6.91	0.0285
9–12 years	0.26	0.03, 2.15	0.2111	0.24	0.03, 20	0.1871
12–15 years	0.95	0.20, 4.48	0.9496	0.84	0.16, 4.33	0.8337
Cohort						
Junju	1.00	.	.	1.00	.	.
Ngerenya early	6.66	3.37, 13.16	<0.0001	2.74	1.19, 6.30	0.0175
Ngerenya late	1.6	0.53, 4.76	0.4017	0.85	0.29, 2.45	0.7579
Number of malaria episodes[i]	1.09	0.90, 1.33	0.3743	0.95	0.78, 1.16	0.5967
Number of malaria episodes in the prior survey	0.88	0.53, 1.46	0.6268	0.81	0.43, 1.51	0.5020
Gametocyte positive in the prior survey	2.56	0.58, 11.30	0.2135	1.59	0.34, 7.34	0.5548
Asexual parasite positive in the prior survey	0.45	0.21, 0.96	0.0398	0.55	0.25, 1.22	0.1392
ABO blood group						
A and AB	1.00	.	.	1.00	.	.
B	1.95	0.82, 4.66	0.1319	1.38	0.56, 3.42	0.4867
O	1.08	0.52, 2.24	0.8393	0.93	0.45, 1.92	0.8399

[i] The sum of the number of malaria episodes occurring in the period leading up to a cross-sectional survey (corrected for the interval between days).
P values in bold are statistically significant (<0.05).
CI – confidence interval.
N/A - sample size insufficient for analysis.

3.6 Discussion

This analysis aimed to describe the prevalence and distribution of gametocyte carriage over time and varied transmission intensities using data collected from a longitudinally monitored cohort of children. Additionally, features predictive of gametocyte carriage were investigated for use (together with insights gleaned from **Chapter 2**) in further work aimed at analysing the dynamics of naturally acquired transmission-blocking immunity (**Chapter 5**). Three cohorts located in sublocations of Kilifi County were used, being: Ngerenya, Chonyi and Junju followed up for 19, 3, and 12 years respectively. The cohorts represented a low to moderate transmission setting (Ngerenya), a moderate (Junju) and a high transmission setting (Chonyi). During the 19-year period of follow up, Ngerenya transitioned from a moderate transmission setting to a low transmission setting. I thus subdivided Ngerenya into two cohorts for the analysis: Ngerenya early (moderate to high transmission – 1998 to 2001) and Ngerenya late (low transmission – 2002 to 2016), giving a total of four cohorts that were eventually used in the analysis.

Parasite prevalence (both gametocytaemia and asexual parasitaemia) appeared to decrease over time in Ngerenya early, Chonyi, and Ngerenya late cohorts. The apparent decrease was in line with reports of declining malaria transmission along the Kenyan coast since 1998[158,461]. These studies analysed parasite prevalence and paediatric malaria admissions in the region over time. The decline in parasite prevalence in the KMLC could be attributed to 1) participation in ITN distribution campaigns from as early as 1993 and 1994, that continued in 2004 (maternal and child welfare clinics), 2006 (first door-to-door campaigns), and 2012 (large-scale community distribution of ITNs), 2) participation in the longitudinal cohort that allowed participants to benefit from better and prompt management of clinical disease, and 3) The change from CQ and SP, following the emergence of drug resistance, to ACTs as first-line therapy for the treatment of malaria.

Despite this overall decline, there was evidence of a resurgence in malaria on the Kenyan coast following the dip in 2009-10[158,461]. In contrast to Ngerenya and Chonyi, parasite prevalence remained mostly constant in Junju, indicating sustained transmission. Malaria hotspots contribute to heterogeneity in transmission[431], and this

is evident in the KMLRC where transmission intensity remains high in Junju despite declining to almost non-existent levels in Ngerenya.

National treatment guidelines for malaria in Kenya recommended CQ use between 1970 to 1999 before SP introduction. SP was then in use until late in 2006 before eventual replacement by ACTs. In Chonyi and Ngerenya early, treatment of malaria was with CQ and SP[461], both of which have been associated with increased post-treatment gametocyte carriage[67,436,437]. Conversely, in Junju the antimalarial in use for the period included in this study was ACTs, particularly artemether-lumefantrine. Artemether-lumefantrine has been described to reduce gametocyte carriage post-treatment[13,77,104]. ACT use may thus explain both the markedly low gametocyte prevalence in Junju and also explain the lack of association between prior clinical episodes of malaria and gametocytaemia as observed in the pre-and post-ACT analysis.

The use of antimalarials in mass drug administration programmes is suggested as a tool for malaria control and has proven efficacious in reducing transmission intensity[110–112]. From this analysis, it appeared that the use of ACTs to treat clinical episodes of malaria was associated with a substantial, cohort-wide reduction in gametocyte carriage. Similar findings have been reported in studies analysing post-treatment gametocyte carriage[462–464]. The impact of ACTs on gametocytaemia in this study is further supported by the disrupted association between malaria and gametocyte carriage post-ACT introduction (**Table 3.8**).

Unfortunately, monitoring data from the Chonyi cohort is available pre-ACT introduction and post-ACT introduction for the Junju limiting the pre-and post-ACT introduction analysis. Further investigation would be required to confirm this finding. Additionally, though monitoring data from Ngerenya cohort is available from 1998 to 2016, parasite prevalence is uncommon after 2006. Nonetheless, Junju and Chonyi sublocations are in proximity to each other (**Figure 3.1**) with residents of a similar background[451], and thus it is likely that similar malaria parasite populations circulate due to the constrained geographical space[430]. For this reason, it would seem more likely that ACT use explains the changing epidemiology of gametocyte prevalence rather than ecological differences between the settings.

Aside from the association observed between malaria episodes and gametocyte carriage, asexual parasitaemia and age also appeared predictive of gametocyte carriage. Consistent with other studies, gametocyte carriage appeared higher in children under five years of age than in the older children as seen in the higher transmission settings (Ngerenya early and Chonyi) where gametocyte prevalence was highest[67–70,459,465]. Consistent with the literature, a higher likelihood of gametocytaemia was observed with concurrent asexual parasitaemia in this analysis[77,459,465]. Interestingly, gametocyte carriage was more strongly associated with asexual parasite positivity at lower transmission intensities (Junju and Ngerenya late) in comparison to higher transmission intensities (Ngerenya early and Chonyi). Presence of gametocytes in the absence of microscopically-detectable asexual parasites may indicate sub-patent asexual parasitaemia[466]. This could be explained by the fact that slower acquisition of immunity to malaria in low transmission settings[467] would predispose individuals to high-density asexual parasitaemia. High parasite densities have been linked to a higher likelihood of gametocytaemia[77,459,465].

Furthermore, the predictive models showed an indication for certain individuals being at a higher risk of gametocyte carriage. Being gametocyte-positive in the prior survey predisposed a subject to gametocyte carriage in the current survey. From their study in Senegal, Grange *et al*. (2015) described hotspots of gametocyte carriage that were associated with active malaria transmission[68]. 'Gametocyte hotspots' could exist in the cohorts under study for this analysis. Studies on the distribution of asymptomatic asexual parasitaemia as well as clinical malaria cases indicate that certain individuals are at a greater risk of [re-]infection[129,433,468–471]. As gametocytes develop from asexual progenitors, it would follow that such individuals may also be predisposed to gametocyte carriage. However, as such individuals may harbour gametocytes at submicroscopic levels, spatiotemporal analysis combined with molecular parasite detection would be required to interrogate the existence of 'gametocyte hotspots'.

There has been evidence to suggest that genetic polymorphisms that protect against severe malaria, such as blood group B and O status and sickle cell trait, are associated with an increased likelihood of gametocytaemia[68,79–81,472]. On the other hand, a precise mechanism that triggers increased gametocyte production in non-B and O blood groups has not yet been defined. In this analysis, however, there was no evidence for an increased likelihood of gametocyte carriage in study participants with sickle cell

or α-thalassemia traits or B and O blood groups. Lamptey *et al*. (2019) similarly found no association between α-thalassemia trait and an increased likelihood of gametocyte carriage[473]. Further investigations into the role of human genetic polymorphisms in influencing gametocytaemia are therefore warranted.

3.6.1 Limitations

One limitation of this study is that parasite detection was by microscopy hence not accounting for sub-microscopic infections. Sub-microscopic gametocytaemia is an essential contributor to the infectious reservoir in malaria-endemic areas[72,474]. Goncalves *et al*. (2017) showed that a substantial proportion of mosquito infections, between 45-75%, are attributable to sub-patent gametocyte carriage[72]. Furthermore, this study[72], as well as other studies employing molecular parasite detection methods[444,445], demonstrated that parasite prevalence was highest in 5 – 15-year olds. Continued parasite exposure, as well as a more developed immune system, may have aided the development of anti-parasite immunity in these older children allowing them to control parasite densities to below the threshold detectable microscopically. Therefore, interventions aimed at reducing transmission may need to target a broad range of age groups to be successful. Additionally, to better capture the infectious reservoir, epidemiological studies need to employ research-grade microscopy or molecular parasite detection techniques such as quantitative PCR[475].

Another limitation of this analysis is the indirect assessment of gametocyte carriage as the microscopy protocol was mainly for evaluating asexual parasite carriage. Consequently, there were more fields examined in blood films where asexual parasites were absent, potentially increasing the chances of detecting gametocytes. The opposite was observed, however, with more gametocytes detected in blood films with asexual parasites. Additionally, the cohorts employed the same microscopy protocol over time and noted associations with gametocytaemia consistent with the literature were observed despite accounting for asexual parasitaemia in the multivariable models. Therefore, the microscopy protocol used did not appear to confound the associations seen between various covariates and gametocytaemia.

3.6.2 Summary of overall findings

These analyses confirm concurrent asexual parasitaemia, age, transmission intensity and prior episodes of clinical malaria as important predictors of microscopically

detectable gametocytaemia. By using three different longitudinal cohorts with a range of transmission intensities, the data confirm the independence and interaction of these factors, making them useful prognostic indicators of transmission that could be used to target transmission-blocking interventions. However, to refine our understanding of the infectious reservoir, epidemiological studies need to employ sensitive parasite detection methods and incorporate assays to measure infectiousness in all age groups across different transmission settings.

A notable finding from these analyses is that the use of ACTs for the treatment of febrile malaria in this cohort of children may have impacted gametocyte carriage. This possibly led to a disruption of the link between malaria episodes and post-treatment gametocyte carriage. While this finding requires further investigation, it could provide preliminary data to support a role for ACTs in reducing malaria transmission.

Chapter 4
Identification and production of *Plasmodium* gametocyte, gamete and ookinete antigens

4.1 Introduction

The development of high-throughput technologies has ushered in the "omics era" generating a vast amount of biological information. Information on the entire genetic sequence of organisms (genomics), transcriptional profiles (transcriptomics), as well as protein expression profiles (proteomics), provides a particularly useful resource for drug or vaccine candidates discovery[476]. In particular, studies analysing genetic variation[355] as well as stage-specific transcription[477,478] and proteome profiles[334,361,479] over the parasite's lifecycle can provide a wealth of data to inform rational vaccine design. Data from such analyses is now publicly available and provides useful criteria for the identification and down-selection of promising vaccine candidates.

Proteomic analyses of the sexual stages have identified several proteins expressed during development within the human host[359–361] and the anopheline vector[362]. Using proteomic data, Stone *et al*. (2018) identified novel TBV candidates, supporting the use of this approach to reveal new targets for TBV development[74]. To rapidly synthesize and evaluate their antigens, the authors used an *E. Coli* in-vitro transcription and translation (IVTT) system to produce protein fragments of their target antigens. The limitation of this approach is that the IVTT system is typically unable to produce complex proteins in their native conformation. This could result in false negatives during screening for transmission-blocking potential. Additionally, the authors expressed only fragments rather than the full-length proteins which potentially restricts the number of epitopes displayed.

P. falciparum proteins are notoriously difficult to produce in heterologous systems[480,481] because of the A+T rich genome, comprising low complexity regions of homopolymeric or heteropolymeric repeats[482–484], and the relatively large size of the proteins[485]. This is exemplified by extensive efforts to produce correctly folded recombinant Pfs230 protein. Pfs230 is a cysteine-domain rich protein whose intricate disulphide-bonding pattern yields a complex tertiary structure [486,487] refractory to full-length synthesis. For this reason, various expression platforms have been evaluated to produce *P. falciparum* proteins for vaccine candidate discovery. These have ranged from bacterial expression

systems (*E. coli*[480] *and Lactococcus lactis*[415]*)* to eukaryotic systems such as the baculovirus[480], mammalian[266,488] and plant-based (*Nicotiana benthamiana*[489]) expression systems. Different expression platforms perform variably with different proteins, and hence the evaluation of more than one platform may be required when faced with particularly challenging proteins[490].

In this chapter, I sought to identify a set of antigens from the gametocyte, gamete and ookinete stages of the parasite for immunoprofiling (chapter 5) and evaluation as potential transmission-blocking vaccine candidates (chapter 6). For this, I mined *P. falciparum* (for gametocyte antigens) and *P. berghei* (for gamete and ookinete antigens) proteomic datasets for novel antigens. I down-selected proteins based on commonly used criteria for surface expression such as the presence of signal peptides, transmembrane (TM) domains, and glycosylphosphatidylinositol (GPI) anchors[74,304]. Additionally, I explored the presence of sequence variation between a reference *P. falciparum* isolate (3D7) and a fully sequenced field isolate (PfKE04) intending to express variants for further analysis. I aimed to produce full-length ectodomains of the identified proteins where possible and chose to use various heterologous expression platforms, mammalian, wheat germ, and *E. coli*, to maximise the likelihood of success in producing the antigens.

4.2 Rationale

In order to capitalise on the vast *P. falciparum* -omic data available, I sought to identify potential vaccine candidates using data from published proteomic datasets[361,364]. To refine the list of antigens, I used publicly available databases, such as PlasmoDB and the Rodent Malaria genetically modified Parasites Database (RMgmDB), hosting various *in silico* prediction algorithms to identify features predictive of surface localisation and probable antigenicity. For the gamete and ookinete antigen selection, antigen selection was based on the rodent parasite *P. berghei*, as it is more amenable to the production of gametes and ookinetes for laboratory assays.

One major obstacle to the development of TBVs for clinical testing from the first identified candidate antigens (Pfs230, Pfs48/45 and Pfs25) was the challenge of producing appropriately immunogenic full-length recombinant proteins. Therefore, I employed three different protein expression systems to increase the chances of producing functional recombinant antigens. I used the wheat germ cell-free system (WGCFS), the mammalian (human embryonic kidney cell line 293E (HEK293E))

system and a bacterial expression system (Rosetta-gami™). The WGCFS has successfully been used to express *P. falciparum* genes without codon-optimisation, producing high yields of correctly folded, soluble protein[491]. Protein expression success rates reported using this system are between 75%[492,493] and 95%[494]. Additionally, the system offers a high-throughput, rapid and relatively simple procedure for protein production and hence is an ideal starting point to evaluate multiple proteins.

Where proteins did not express in the wheat germ system, I used either the HEK293E or Rosetta-gami™ expression platforms. The HEK293E expression platform[495] has been used to produce gametocyte antigens with a success rate of around 68%[304]. For expression in the HEK293E system, sequences corresponding to the gene of interest (GOI) were first codon optimised to overcome the A+T bias of *P. falciparum* genes, and potential N-glycosylation sites were modified to avoid masking of potential critical epitopes. Crosnier *et al.* (2011)[485] showed that these approaches could improve protein production. Though bacterial expression systems have a reportedly low success rate when producing eukaryotic proteins[481], optimised bacterial strains have been developed to overcome this challenge. I chose the Rosetta-gami™ bacterial strain for this work as mutations in the glutaredoxin reductase and thioredoxin reductase genes promote disulphide bond formation thereby promoting protein folding[496,497]. Additionally, the bacteria are supplemented with rare tRNAs to overcome codon bias during heterologous protein expression. By combining these three platforms, I anticipated expressing a minimum of eight antigens from each stage (gametocyte and gamete/ookinete stages) for further assays.

4.3 Objectives

The main objective was to identify sexual stage antigens with potential as TBV candidates and produce them as recombinant protein for further immunological (**Chapter 5**) and functional (**Chapter 6**) characterisation.

4.3.1 Specific Objectives

- Identify potential TBV candidate antigens highly expressed in mature gametocyte, gamete, and ookinete stages.
- Analyse sequence variation in the antigens identified between the reference isolate 3D7 and a local field isolate.

- Produce protein corresponding to the antigens identified at high enough yield for use *in vitro* and *ex vivo* assays.

4.4 Materials and Methods

4.4.1 Materials

A summary of commercially available reagents is provided in **9.4 Appendix 4,** while the recipes for the buffers and solutions used in this study are provided in **9.5 Appendix 5**.

4.4.2 Antigen identification and prioritisation

4.4.2.1 Gametocyte antigens

To identify sexual stage antigens enriched in mature gametocytes that may be targets of naturally acquired antibodies, I used a published *P. falciparum* gametocyte proteome dataset from Lasonder *et al.* (2016)[361]. The data contained 2,241 proteins identified from a mixed culture of *P. falciparum* male and female gametocytes. A shortlist of 430 antigens was selected by enriching the published dataset for proteins highly expressed in stage V gametocytes. This was done by selecting a cut-off expression value (greater than or equal to 50%) based on the expression values of the known TBV antigens, Pfs48/45 (53.3%) and Pfs230 (63.6%) which were considered the 'gold standard'. The data were then sorted by their gene description, and 333 predicted intracellular proteins were manually filtered out. These included ribosomal proteins, intracellular enzymes and transcription factors. The remaining list of 97 proteins was combined with a list of 16 potential molecular biomarkers of gametocytaemia previously identified (Kapulu M, personal communication). These potential molecular biomarkers were downselected from a previously identified list using mRNA expression data from published datasets[363,477,498–501].

I then searched the 113 gene IDs corresponding to these proteins on PlasmoDB (Release 31, 9th March 2017), GeneDB (https://www.genedb.org/), Tropical Diseases Research (TDR) Targets Database (Release 5, https://tdrtargets.org/) and the rodent malaria genetically modified parasites database (RMgmDB, https://www.p*berghei*.eu/index.php). PlasmoDB is a comprehensive database of *Plasmodium* "genomic, transcriptomic, proteomic and metabolomic data"[502] readily accessible for data mining. Similarly, GeneDB is a prokaryotic and eukaryotic

pathogen database that is manually curated, and for this work provided complementary data to PlasmoDB. TDR targets, on the other hand, is an online resource that integrates genomic and functional data to provide a platform for the identification of pathogen targets for drug or vaccine development[503]. RMgmDB is a database of *P. berghei* gene knockout studies from across the parasites developmental stages[504]. A description of phenotypes arising from the gene disruption is also provided. Information on the presence of signal peptides (SP), transmembrane domains ™, GPI anchors, protein export motifs, predicted antigenicity and possible evidence for a role in gametocyte/gamete development or fertilisation (based on rodent malaria gene disruption studies) was then recorded for each antigen. All proteins with a signal peptide were then selected, and a shortlist of 24 candidate antigens prioritised for protein production. These proteins were mainly selected for not previously being studied, avoiding duplication of effort. One additional novel antigen was identified from a conference abstract[282] to give a total of 25 proteins. A summary of the selection process with the number of proteins identified in each step is provided in (**Figure 4.1**). Pfs230 and Pfs48/45 were included in the list as "gold-standard" antigens as they are leading gametocyte-expressed TBV candidates.

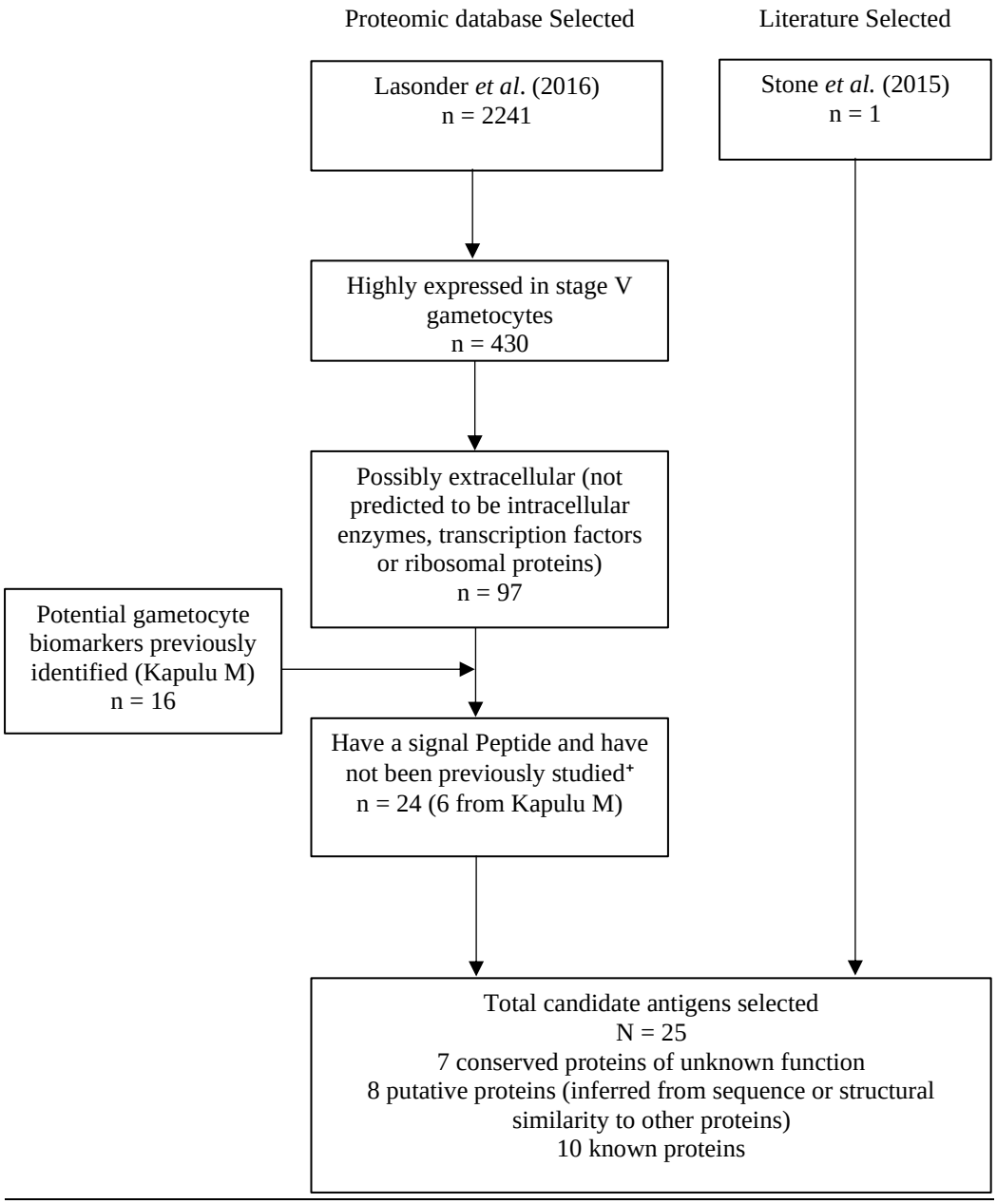


Figure 4.1: Flow diagram showing the selection of candidate gametocyte antigens for study. The number of proteins at each step is also indicated.
[+]Had not been studied as TBV candidates, based on studies in *P. falciparum*, at the time of the search.

4.4.2.2 Gamete and ookinete antigens

For this objective, the rodent parasite *P. berghei* was used as it provides a reliable, high-throughput, and efficient means to study gametes and ookinetes *in vitro*. Study of these antigenic targets in *P. falciparum* is comparatively challenging at the desired throughput (reviewed in[399]). I identified genes potentially expressed on the surface of gametes and ookinetes by bioinformatic analysis of previously published proteome data[364]. A total of 22 candidate genes were identified, 12 of these were identified from the Talman *et al*. (2014) published proteome of the microgamete[364], five were identified by bioinformatic prediction of surface localisation (Blagborough A, personal communication), and a further five from experimental evidence of surface localisation on gametes/ookinetes (Angrisano F, and Blagborough A, personal communication). P28 (*P. berghei* protein in the same gene family as Pfs25) that induces potent transmission-blocking antibodies in mouse models[323] was also included in the list.

To arrive at the 12 potential antigens, I used data from the proteomic analysis of male gametes generated by Talman *et al.* (2014)[364]. In their study, Talman *et al*. carried out three biological replicates for their protein identification. Therefore, proteins that were only identified in one of the biological replicates run during the experiment were first excluded for higher stringency. After this, the proteins were sorted for the presence of a signal peptide, to increase the chance of selecting surface-expressed proteins, and those without were removed from the list. Proteins predicted to have a signal peptide with a score of 0.5 or greater were then included. Known intracellular proteins, known gametocyte proteins and well-studied gamete/ookinete proteins were also then taken out of the list. Additionally, proteins with a protein score less than that of P28 were taken out of the list. Finally, I excluded all proteins with a molecular weight above 120 kDa from the list as these would potentially be challenging to express[505]. The remaining 40 proteins were then searched on parasite databases for features predictive of surface localisation, antigenicity and a role in gamete-to-oocyst formation. Twelve that had not been extensively studied were then prioritised for analysis. A summary of the process of selection is provided in **Figure 4.2**.

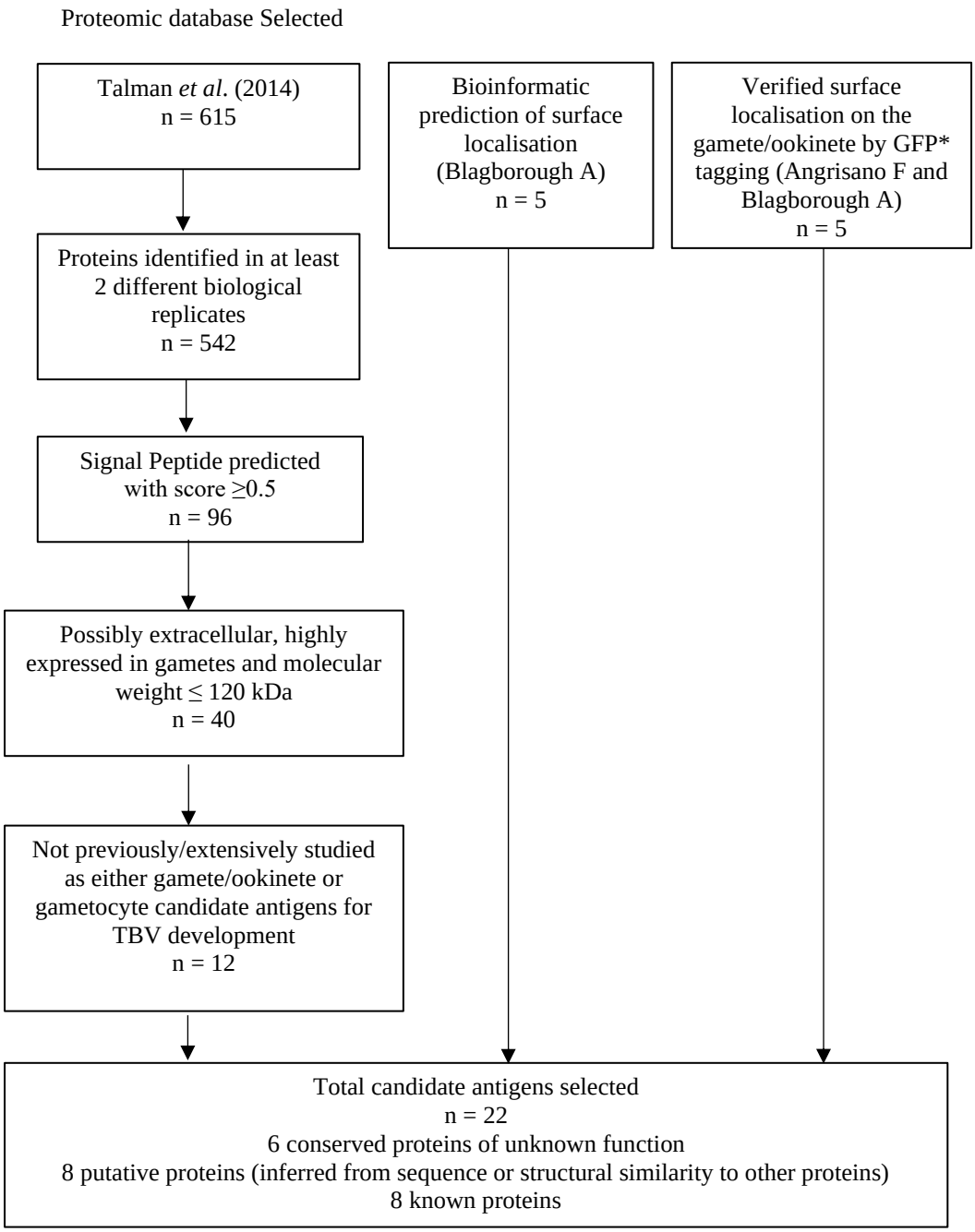


Figure 4.2: Flow diagram showing the selection of candidate gamete and ookinete antigens for study. The number of proteins at each step is also indicated.
*GFP – green fluorescent protein

4.4.3 Parasitology

4.4.3.1 Parasite strains

P. falciparum parasite strains used for this study were 3D7 (provided by Lydia Nyamako, this parasite has been in continuous culture at KWTRP) and the field isolate PfKE04 (provided by Dr Abdirahman Abdi). Additionally, *P. berghei* ANKA strain, clone 2.34 was used (parasite in TRIzol® and genomic DNA were provided by Dr Andrew Blagborough).

4.4.3.2 Parasite culture and preparation of RNA and cDNA

P. falciparum asexual parasites were cultured in complete culture media (**9.5 Appendix 5**) in an incubator at conditions of 92% N_2, 3% O_2, 5% CO_2 and set at a temperature of 37ºC. The parasites were maintained in culture for three weeks to bulk up genetic material. When the cultures reached a minimum of 12% parasitaemia, the parasites were harvested by centrifugation at 500 x g for five minutes. Approximately 200 µl of parasite pellet was resuspended in 1 ml of TRIzol® and stored at -80°C awaiting RNA extraction and cDNA synthesis. *P. berghei* parasite pellets stored in TRIzol® (separately prepared from gamete and from ookinete stage parasites) were provided by Dr Blagborough. Parasite RNA was extracted using the QIAGEN® RNeasy® kit and cDNA prepared using the SuperScript™ III First-Strand Synthesis System according to the manufacturer's instructions.

4.4.4 Molecular biology and cloning

Three different expression systems were used for protein expression (the wheat germ cell-free, mammalian and bacterial expression systems) and hence different expression vectors were used for each of the systems. A summary of the process used to create recombinant plasmids for use in each system is provided in **Figure 4.3**, and a detailed description is provided in the text that follows.

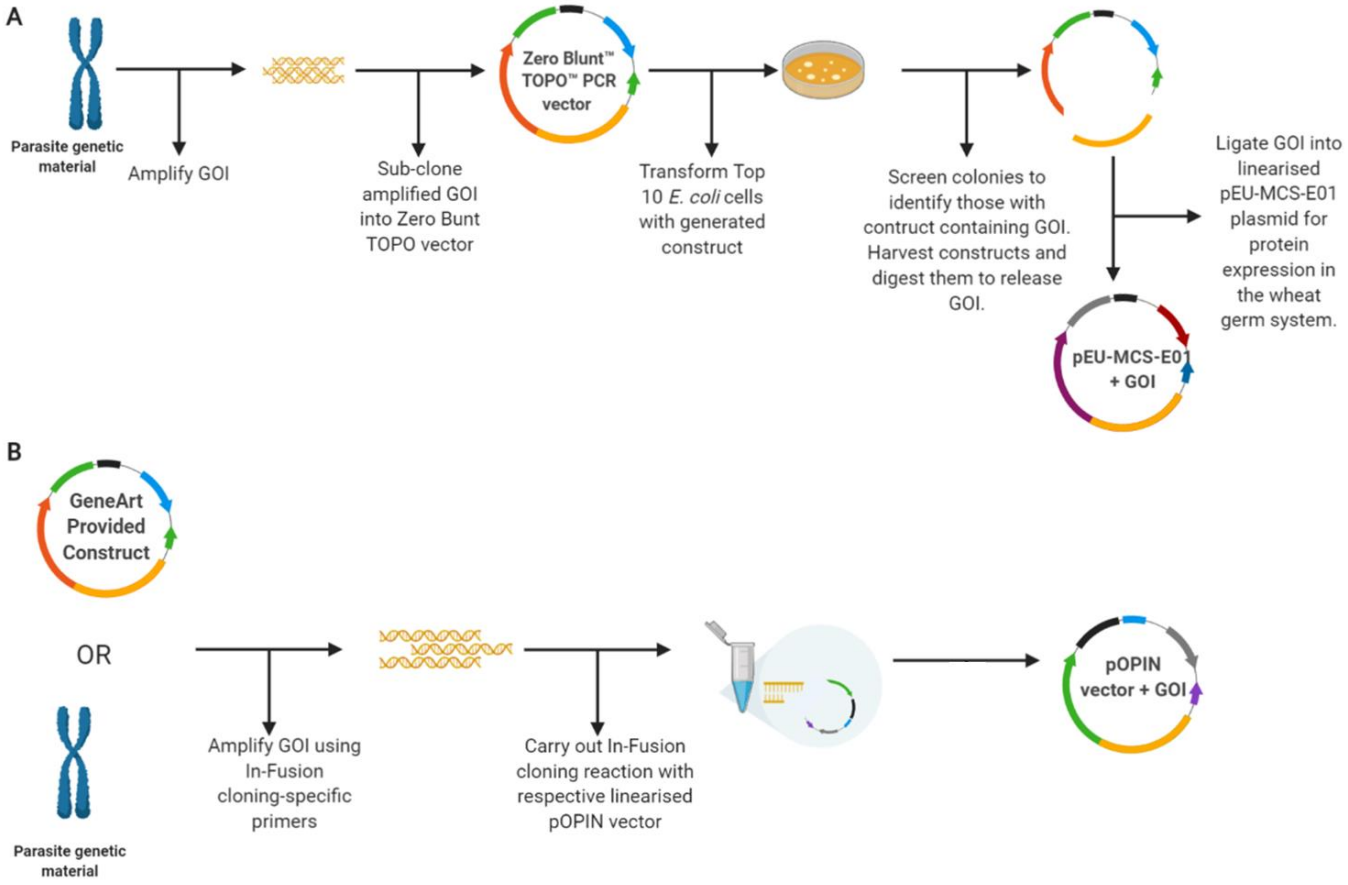


Figure 4.3: Schema showing the cloning strategies employed to generate recombinant constructs for protein expression. (A) Cloning strategy for generating constructs for expression in the wheat germ expression plasmid (pEU-MCS-E01) via sub-cloning into the ZeroBlunt™ TOPO™ ™ PCR vector. (B) Cloning strategy employed to generate constructs for use in either the mammalian or the bacterial expression systems. For the mammalian expression system, respective genes were amplified from commercial constructs containing codon optimised sequences corresponding to the genes of interest (GOI). For the bacterial expression, the genes were amplified from parasite genetic material. Image created using ©BioRender (https://app.biorender.com/).

4.4.4.1 Wheat germ cell-free expression system (WGCFS)

4.4.4.1.(a) Construct design

Protein sequences corresponding to the candidate antigens were obtained from PlasmoDB database (Release 31, 9th March 2017) using *P. falciparum* 3D7 lab strain as the reference genome (version 3.0). In addition to 3D7, I obtained corresponding gene sequences from a fully sequenced field isolate from Kilifi – PfKE04 – (kindly provided by Dr Abdirahman Abdi). I then analysed sequence variation between the two isolates and where there was either an insertion/deletion or non-synonymous single nucleotide polymorphism (SNP) between the 3D7 and PfKE04 sequences both gene versions were included for construct design. Predicted signal peptide sequences, transmembrane domains occurring at the termini of proteins and GPI anchor coding regions were removed from each of the sequences before cloning.

4.4.4.1.(b) PCR amplification

(i) Primer Design

Optimal annealing temperatures for each primer pair was determined using the ThermoScientific online Tm and annealing temperature calculation tool (https://www.thermofisher.com/ke/en/home/brands/thermo-scientific/molecular-biology/molecular-biology-learning-center/molecular-biology-resource-library/thermo-scientific-web-tools/tm-calculator). Polymerase chain reaction (PCR) primers were designed to contain the Xho1 restriction site (or Kpn1 if an internal Xho1 restriction site was detected in the sequence) and a start codon in the forward primer. The Not1 restriction site and a stop codon were included in the reverse primer to correspond to the restriction sites on the WGCFS expression vector, pEU-MCS-E01, multiple cloning site (MCS) (**9.6 Appendix 6**). The reverse primer also contained a hexahistidine coding sequence to allow for affinity purification of the expressed protein using nickel resin. Additionally, flank-to-flank primers were designed based on the sequences upstream and downstream of the MCS. The flank-to-flank primers were used to screen recombinant plasmids harvested from bacterial colonies after cloning to verify that the sequence of interest was present.

(ii) PCR reaction and cycling conditions

PCR was carried out using Phusion® High-Fidelity PCR Master Mix in 20 µl reactions as follows:

Reagent	**Volume (µl)**
2 x Phusion® master mix PCR reagent	10
10 µM forward primer	1
10 µM reverse primer	1
Template DNA	2
Nuclease-free water	6
Total	20

Cycling conditions were as follows: Initial denaturation at 98°C for 30 seconds, followed by 35 cycles of denaturation at 98 °C for 15 seconds, annealing at optimum temperature for 30 seconds, extension at 72°C for 30 seconds. A final extension at 72°C for 5 min was included at the end of cycling, followed by a final hold at 10°C for 10 minutes.

4.4.4.1.(c) Agarose gel electrophoresis

PCR reaction products were run on 0.8% (for DNA fragments greater than 1000 base pairs (bp)) or 1 % (for fragments smaller than 1000 bp) agarose gels prepared in 0.5 x Tris-borate-EDTA (TBE) buffer. The agarose was first melted by boiling in TBE buffer before 1 x RedSafe® gel stain was added to the agarose. The mixture was swirled to mix and poured into a casting tray to solidify. DNA loading dye was then added to the PCR products to a final concentration of 1 x and loaded onto the gel alongside Hyperladder I DNA marker and the gel run for one hour at 100 volts.

4.4.4.1.(d) Gel extraction and purification of DNA

Bands corresponding to the predicted PCR product size were excised from the agarose gels after electrophoresis and DNA purified from the excised gels using QIAquick kit according to manufacturer's instructions. DNA was eluted at the final step in 30 µl of pre-warmed elution buffer provided in the kit and quantified using a NanoDrop 1000 spectrophotometer.

4.4.4.1.(e) Sub-cloning into Zero Blunt™ TOPO™ ™ PCR vector

Prior to cloning into the pEU-MCS-E01 vector, purified PCR products for wheat germ cell-free protein expression were first sub-cloned into the Zero Blunt™ TOPO™ PCR vector (**9.6 Appendix 6**) according to manufacturer's instructions. The Zero Blunt™ vector was used as it offers a rapid and efficient system for the cloning of blunt-ended PCR products via a *Vaccinia* virus DNA Topoisomerase 1-mediated cloning reaction[506]. Recombinant plasmids from the ligation reactions were then propagated in *E. coli* cells.

4.4.4.1.(f) Transformation and growth of bacterial cells

ONE SHOT™ TOP 10 *E. coli* cells were transformed with recombinant plasmids from the cloning reactions by heat shocking 25 µl of the competent cells in the presence of 1 µl of plasmid at 42°C for 30 seconds. The transformation mix was then placed on ice for two minutes before 250 µl of super optimal broth with catabolite repression (SOC) media was added, and the cells incubated at 37 °C in an incubator to set to shake at 225 revolutions per minute (rpm). The transformed bacterial cells were then plated onto lysogeny broth (LB)/Agar plates containing kanamycin at 50 µg/ml and incubated at 37 °C overnight. The next day, a minimum of 5 single colonies were picked and screened for the insert by colony PCR.

4.4.4.1.(g) Colony PCR reaction and cycling conditions

Colony PCR was carried out to screen bacterial colonies using the KAPA2G Fast HotStart ReadyMix kit. A single colony was picked from an LB/Agar plate and diluted in 10 µl of nuclease-free water prior to setting up the PCR reaction as follows:

Reagent	**Volume (µl)**
2 x KAPA2G Fast HotStart ReadyMix	12.5
10 µM forward primer	1.25
10 µM reverse primer	1.25
Template DNA	1
Nuclease-free water	9
Total	25

Cycling conditions were as follows: Initial denaturation at 95°C for 3 minutes, followed by 35 cycles of denaturation at 95°C for 40 seconds, annealing at optimum

temperature for 60 seconds, extension at 68°C for 3 minutes. A final extension at 72°C for 5 min was included at the end of cycling. M13 forward and reverse primers were used as they flanked the inserted gene of interest. PCR products were run on agarose gels as described to determine whether the GOI integrated into the vector, and identify the colonies containing bacteria with the recombinant vector. The gene fragments for cloning were then excised from the successful recombinant vectors by restriction digestion, separated out from the vector backbone by gel electrophoresis before being recovered and purified by gel extraction.

4.4.4.1.(h) Plasmid recovery for protein expression

Colonies from bacteria containing plasmids with the antigens of interest were then grown in 3 ml of LB broth containing kanamycin. The next day bacteria from the culture were pelleted, and plasmids extracted from the bacteria using QIAprep® Spin Miniprep Kit according to the manufacturer's instructions. Where higher concentrations of plasmid were required, 1 ml of the 3 ml overnight culture was used to inoculate 100 ml of fresh LB broth. This culture was then left to grow overnight, and on the next day, plasmids were extracted from the bacteria using QIAGEN® Plasmid Plus Maxi Kit according to the manufacturer's instructions.

4.4.4.1.(i) Restriction digestion

Restriction digestion reactions were set up to digest out fragments from the Zero Blunt™ TOPO™ PCR vector for cloning into the pEU-MCS-E01 plasmid and linearization of the pEU-MCS-E01 plasmid for cloning as follows:

Reagent	**Volume (µl)**
DNA	A volume of DNA corresponding to a maximum of 1 µg DNA
10 x Cutsmart Buffer	1
10 µM reverse primer	5
Restriction enzyme 1	1
Restriction enzyme 2	1
Nuclease-free water	Top up to final volume 50 µl

The reaction was then incubated at 37 °C for 1 hour.

4.4.4.1.(j) Cloning into the pEU-MCS-E01 expression vector

For cloning into the pEU-MCS-E01 vector, optimal vector: insert ratios were calculated based on the molar mass of the respective DNA fragments (linearised vector and purified gene fragment) using the New England Biolabs (NEB) online ligation calculation tool (https://nebiocalculator.neb.com/#!/ligation). Ligation was carried out using the Ligation High Ver.2® reagent at 16°C for one hour in a thermocycler. Negative control reactions using a linearised vector with no insert were also set up to test for background colonies resulting from bacteria that had taken up the undigested vector. One Shot™ TOP 10 *E. coli* cells were transformed with recombinant plasmids from the cloning reactions as described **4.4.4.1.(f)**. Colony PCR was carried out as described (**4.4.4.1.(g)**) using flank-to-flank primers that were designed to span the regions upstream and downstream of the inserted GOI within the pEU-MCS-E01 vector (**9.6 Appendix 6**). The PCR products were run on agarose gels (**4.4.4.1.(c)**) to identify bacterial colonies containing recombinant vectors prior to plasmid extraction (**4.4.4.1.(h)**). Plasmid purity and concentration were then measured spectrophotometrically (NanoDrop 1000). Plasmids used for protein expression had a purity in the range of $A_{260/280}$ 1.80 – 1.89.

4.4.4.2 Mammalian expression system (Human embryonic kidney cell-line E (HEK293E))

4.4.4.2.(a) Construct design

For gametocyte antigens targeted for expression using the HEK293E mammalian expression system, I replaced the endogenous signal peptide sequence with the human tissue plasminogen activator (tPA) signal sequence. The tPA sequence has been shown to enhance heterologous protein expression in eukaryotic expression systems[507–509]. GPI anchor sequences were also removed as differences between mammalian and plasmodial GPI-anchoring requirements can affect the production of soluble protein[510,511]. To express only the extracellular portion of the protein, I also took out transmembrane domains where possible. Moreover, as *N*-linked glycans are thought to be mostly absent in *P. falciparum*[512,513], potential N-glycosylation sites were identified using the NetNGlyc prediction algorithm[514] (http://www.cbs.dtu.dk/services/NetNGlyc/). Predicted glycosylation sequons (Asn-X-Thr/Ser) with a threshold above 0.5 were modified by substituting asparagine

residues to glutamine residues (Gln-X-Thr/Ser). Furthermore, at the 5' end of the tPA signal sequence, a Kozak consensus sequence (5'-CCACC-3') was added upstream of the ATG initiation codon site for enhanced translation[515], and a stop codon added at the end. The sequences were then sent to GeneArt® (Life Technologies, Germany) for codon optimisation to overcome the adenine and thymine bias in *P. falciparum* genes[482].

4.4.4.2.(b) PCR amplification

(i) Primer design

For protein expression in the HEK293E system, In-Fusion® cloning primers were designed to amplify codon-optimised gene constructs from the GeneArt®-provided vectors containing the genes of interest. Primers compatible with In-Fusion cloning into the pOPINGS vector (a gift from Ray Owens, Addgene plasmid # 41121) were designed using the online tool provided by the Oxford Protein Production Facility (OPPF, https://www.oppf.rc-harwell.ac.uk/OPPF/). The primers were designed to contain 15 base pair overhangs compatible with cloning into the pOPINGS vector (**9.6 Appendix 6**).

(ii) PCR reaction and cycling conditions

PCR was carried out using Phusion® High-Fidelity PCR Master Mix as described in **4.4.4.1.(b)(ii)** using as template the GeneArt®-provided vectors.

4.4.4.2.(c) Generation of recombinant plasmids by In-Fusion cloning

Purified PCR products (**4.4.4.1.(d)**) derived from amplifying the respective gene fragments from GeneArt® provided constructs were ligated into the linearised pOPINGS vector using the In-Fusion cloning kit at a 2:1 insert to vector ratio. Volumes of insert and vector to use for a 2:1 ratio were again determined using the NEB online ligation calculation tool. Ligation reactions were set up as follows:

Reagent	Volume (µl)
5 x In-Fusion® HD enzyme premix	2
Linearised vector	1
Purified PCR product	variable
Nuclease-free water	Top up to final volume 10 µl

The reaction was then incubated at 50°C for 15 minutes before being placed on ice awaiting transformation. Negative control reactions using linearised pUC19 vector with no insert and positive control reactions using linearised pUC19 vector plus control insert were also set up. Ligations were then transformed into ONE SHOT™ TOP 10 *E. coli* cells as described (**4.4.4.1.(f)**) and the next day colonies screened for recombinant plasmid using the T7 promoter forward primer, and an antigen-specific reverse primer as described (**4.4.4.1.(g)**). Recombinant plasmids were then harvested for use in protein expression using the QIAGEN® Plasmid Plus Maxi Kit as described (**4.4.4.1.(h)**).

4.4.4.3 Bacterial expression system (Rosetta-gami™)

4.4.4.3.(a) Construct design

Constructs were designed similar to constructs for the WGCFS as described in **4.4.4.1.(a)**.

4.4.4.3.(b) PCR amplification

(i) Primer design

For protein expression in the Rosetta-gami™ bacterial expression system, In-Fusion® cloning primers corresponding to the pOPINS3C vector (also a gift from Dr Ray Owens (**9.6 Appendix 6**)) –Addgene plasmid # 41115 - were designed using the OPPF online tool. Flank-to-flank primers were also designed for colony PCR.

(ii) Gene amplification PCR reaction and cycling conditions

PCR was carried out using Phusion® High-Fidelity PCR Master Mix as described (**4.4.4.1.(b)(ii)**) using parasite genetic material as a template.

4.4.4.3.(c) Generation of recombinant plasmids by In-Fusion® cloning

Purified PCR products were ligated into the linearised pOPINS3C vector using In-Fusion® cloning as described (**4.4.4.2.(c)**). Recombinant pOPINS3C plasmids containing the antigens of interest were transformed into Rosetta-gami™ B(DE3) pLysS competent cells as described (**4.4.4.1.(f)**). Colony PCR using pOPINS3C flank-to-flank primers was then used to identify positive colonies (**4.4.4.1.(g))**. A single colony was used to inoculate 3 ml of fresh terrific broth containing 100 µg/ml carbenicillin in order to generate a starter culture for use in protein expression. Terrific broth was used as it is a richer broth than LB, supporting higher bacterial cell density for optimal protein expression[496].

4.4.4.4 Plasmid sequencing for verification (MiSeq and Sanger Sequencing)

Plasmids were sequenced to verify protein identity and to confirm that no mutations had been introduced during the cloning process as they could potentially affect protein structure. Two sequencing platforms, the Illumina MiSeq and Sanger sequencing, were used based on the facilities available at the time of recombinant plasmid production.

4.4.4.4.(a) Illumina MiSeq platform

Plasmids were first linearised using a single restriction enzyme (see **4.4.4.1.(i)**) before they were sent to the in-house sequencing facility at KWTRP. The standard sequencing protocol developed at the facility was followed. Briefly, the Nextera® XT kit was used for library preparation as per manufacturer's instruction. First, the sequences were fragmented, and adaptors ligated to them after which limited PCR was carried out to add indexes to the sequence for identification. Products from the PCR reaction were then cleaned up using AMPure® beads (0.6 x); during this step, small fragments below 300 bp were eliminated. The fragment size was then determined using a bioanalyser and samples quantified using a Qubit® 2.0 fluorometer. All samples were then normalised to 2 nM concentration before being pooled together and denatured using 0.2 M sodium hydroxide. The concentration of the pooled samples was then adjusted to 8 picomoles, before being spiked with PhiX (in-house sequencing positive control) and denatured at 96°C for two minutes. The

pooled samples were then loaded onto the MiSeq machine for sequencing. The quality of the generated reads was analysed using the MultiQC tool (version 1.4)[516] before the reads were assembled and mapped to each respective reference sequence using Geneious sequence analysis software (version 11.1.2).

4.4.4.4.(b) Sanger Sequencing

Sanger sequencing was outsourced to GENEWIZ®, a genomics company located in Essex (United Kingdom) and was carried out using the flank-to-flank primers used for colony PCR. Each plasmid was sequenced in the forward and the reverse direction and the resultant reads aligned onto the reference sequence using the MUSCLE alignment algorithm present in Geneious sequence analysis software. Where possible, if sequence variation was observed in both the forward and reverse reads, a second plasmid batch from a different colony was sequenced.

4.4.5 Recombinant protein production

4.4.5.1 Wheat germ cell-free expression system (WGCFS)

4.4.5.1.(a) Protein Expression

Protein expression was carried out using the WGCFS WEPRO® 7240H kit. Protein expression was carried out as a 2-step process with transcription and translation carried out in separate reactions. Proteins were first expressed in small-scale reactions, and where protein expression was confirmed, the production scale was increased to mid-scale or large-scale depending on the yield obtained. The transcription reaction was set up as follows:

Reagent	**Final Concentration**
Plasmid DNA (1 µg/ul)	100ng
25 mM dNTP mix	2.5 mM
5 x translation buffer	1 x
RNase inhibitor (800 U/µl)	1 U/µl
SP6 RNA polymerase (800 U/µl)	1 U/µl
Nuclease-free water	Top up to final volume 20, 50 or 250 µl (for small-, mid- or large-scale respectively)

The reaction mixture was then incubated at 37°C for 6 hours after which an aliquot of the generated mRNA was run on a 1% agarose gel to check for mRNA degradation before translation was carried out. The translation reaction was set up as below for small-scale protein expression:

Reagent	**Final concentration**
mRNA	0.5 x
Creatine kinase (20 mg/ml)	40 ng/µl
WEPRO® 7240H (240 OD/ml)	120 OD/ml
Total	500 µl

Translation was carried out in a bilayer reaction format with the mRNA/wheat germ extract (WGE) mix in the lower layer and the translation substrate in the upper layer. The translation reaction was carried out at 15°C for 20 hours, and the translation mix transferred to 4°C immediately after preparation to await protein purification.

4.4.5.1.(b) Protein purification

Imidazole to a final concentration of 50 mM was added to the translation mix or culture supernatant (WGCFS and mammalian expression system, respectively). An appropriate volume of nickel resin (Ni Sepharose® High Performance (HP) affinity resin) equilibrated in binding buffer was then added to the translation mix or culture supernatant. Binding of the protein to the nickel resin was achieved by incubating the tube containing the protein/resin mix on a rotating platform for 2 hours at 4 °C. The protein/resin mix was then transferred to a disposable polypropylene column fitted with a porous polyethylene frit designed to trap the resin while allowing the solution to flow through. The trapped resin was then washed five times using a volume of binding buffer corresponding to 10 times the volume of resin used. The protein was eluted from the resin using either 20, 100 or 200 µl of elution buffer (depending on the expression scale and expected yield of protein). The required volume of elution buffer was applied five separate times, and each time the eluate was collected into the same tube at final volumes of either 100, 500 or 1000 µl.

4.4.5.2 Mammalian expression system

4.4.5.2.(a) Protein Expression

The Human Embryonic Kidney 293 cell line modified with the Epstein Barr virus nuclear antigen 1 (HEK293E - a kind gift from Dr James Tuju) was used for mammalian expression. The HEK293E cells were grown to a cell density of 1.1 x 10^6 cells/ml with minimum viability of 97% before transfection. Transfection was carried out using Lipofectamine® 3000 reagent. For a 20 ml transfection volume, a volume corresponding to 30 µg of plasmid DNA was added to 2 ml of Opti-MEM™ reduced serum media together with P3000® reagent at a volume corresponding to 2 x the volume of DNA added. This mixture was then vortexed mildly and set aside. To a second tube containing 2 ml of Opti-MEM™, 30 µl of lipofectamine was added and the mixture vortexed mildly before being incubated at room temperature for 5 minutes. After this, the Opti-MEM™/lipofectamine mix was added to the Opti-MEM™/DNA mix and the mixture incubated at room temperature for 20 minutes. This transfection mix was then added to the HEK293E cells, and the flask swirled several times to distribute the DNA complex evenly. The flask containing the transfected cells was then transferred to a humidified incubator set at 37°C with 5% CO_2 supply and shaking set at 130 rpm. Twenty-four hours post-transfection, a casein peptone mix, TN1, enriched with vitamins and growth factor was added to a final concentration of 0.5%. The cells were left to grow for three days before the supernatant was harvested for protein purification.

4.4.5.2.(b) Protein purification

Protein purification and quantification were carried out as described in **4.4.5.1.(b)**.

4.4.5.3 Bacterial expression system

4.4.5.3.(a) Protein expression

Protein expression was carried out in Rosetta-gami™ cells using a 1 in 50 dilution of a starter culture prepared the night before (see section 4.4.3.7) into 500 ml of terrific broth. The new culture was then incubated at 37°C until an optical density (OD) of between 0.5 to 0.8 was reached. The culture was then placed at room temperature to cool before induction of expression using 0.1 mM of Isopropyl β-d-1-thiogalactopyranoside (IPTG). The culture was then incubated at 20 °C overnight in

an incubator set to shake at 225 rpm. The next day, the bacterial cells were pelleted by centrifugation at 5000 x g for 10 minutes, and the pellet set aside for protein purification.

4.4.5.3.(b) Protein purification

Bacterial pellets (refer to **4.4.5.3.(a)**) were first homogeneously resuspended in 5ml of bacterial lysis buffer (**9.5 Appendix 5**) per gram of bacterial pellet. An equal volume of binding buffer was added to the resuspended bacterial pellet as well as imidazole to a final concentration of 50 mM. The bacteria were then lysed by sonication, five rounds of 30 seconds sonication followed by 30 seconds on ice were carried out, after which an appropriate volume of nickel resin (HisPur™ Ni-NTA resin) was added. The protein/resin mix was then placed on a rotating platform at 4 °C for 2 hours to allow binding. The expressed protein was then purified by gravity flow using the same protocol applied for proteins expressed in the WGCFS and mammalian expression systems (**4.4.5.1.(b)**).

4.4.5.4 Protein identification and quantification

Confirmation of protein expression was done by running an aliquot of the purified protein prepared in laemmli buffer, alongside the flow-through and wash fractions, on a denaturing sodium dodecyl sulphate polyacrylamide gel (SDS PAGE) in the tris glycine buffer system. The samples were run at 150 volts until the dye front migrated to the bottom of the gel. Where available, pre-cast Bolt™ 4-12% Bis-Tris Plus gels were also used to separate the purified protein using 2-(N-morpholino) ethanesulfonic acid (MES) buffer at 180 volts for 35 minutes. Protein bands were visualised by Coomassie staining using InstantBlue® rapid stain. The purified protein was quantified using the Bradford Assay with bovine serum albumin (BSA) as the protein standard according to the manufacturer's instruction.

4.4.5.5 Protein identity confirmation

4.4.5.5.(a) Western blot analysis

To confirm that the purified proteins were the expressed antigens, western blot analysis using an anti-histidine antibody (Monoclonal Antibody 3D5 – HRP) was performed. After running the proteins on an SDS-PAGE gel, they were transferred onto a polyvinylidene difluoride (PVDF) membrane by wet transfer at 80 volts for an

hour. The membrane was then blocked for one hour with 4% non-fat skim milk prepared in tris buffered saline (TBS) containing 0.05% Tween 20® detergent (TBS/T) at room temperature. The membrane was then washed three times using TBS/T with a five-minute incubation between each wash. After this, the membrane was probed using an anti-histidine antibody fused to horseradish peroxidase (HRP) and incubated at room temperature for one hour. The membrane was then washed once more with TBS/T before being incubated with Novex® ECL chemiluminescent substrate for 1 min and visualised in a Bio-Rad imager.

4.4.5.5.(b) Mass spectrometry (liquid chromatography (LC) tandem mass spectrometry (MS) - LC/MS/MS

Proteins were prepared for mass spectrometry by first adding 90 µl of 50 mM triethylammonium bicarbonate buffer to 10 µl of protein sample (minimum concentration used was 20 ng). After this, 40 mM of dithiothreitol (DTT) was added and the proteins incubated at 65°C with shaking at 400 rpm for one hour. 80 mM of iodoacetamide was then added, and the samples then incubated in the dark for one hour at room temperature. After this, 80 mM of DTT was added and the samples incubated further for 30 minutes. The samples were then digested using 400 ng of trypsin overnight at 37°C with gentle agitation in a shaking incubator. The next day, the proteins were concentrated using a speed vacuum concentrator to reduce the volume to about 50 µl. Equilibration buffer was then added to a final concentration of 0.1% before the samples were bound to chromatography resin in a Zip Tip® by pipetting 15 µl of the sample through the resin ten times. This Zip Tip® was first pre-wetted in 100% acetonitrile and equilibrated in 0.1% trifluoroacetic acid (TFA) before sample binding. The resin in the Zip Tip was then washed in 0.1% TFA by pipetting up and down seven times to precipitate the protein. The protein was eluted into 20 µl of elution buffer before being concentrated down to between 1 to 2 µl. Finally, 15 µl of loading solution was added to the samples and the samples stored at -80°C awaiting injection into the mass spectrometer. The generated data were searched against the *P. falciparum* protein database using the Mascot algorithm (http://www.matrixscience.com/server.html).

Mass spectrometry analysis was carried out both in-house at KWTRP and, for a subset of proteins, outsourced to the Cambridge Centre for Proteomics (Cambridge, United

Kingdom) for protein identification analysis and de novo peptide sequencing where required.

4.5 Results

4.5.1 Gametocyte antigen production and purification

4.5.1.1 Potential antigens identified for analysis

A list of 24 gametocyte antigens was identified by analysing published proteome data for proteins with high expression in the mature gametocytes and using parasite databases to identify possible surface-localised proteins. An additional potential antigen (NOT1) was identified from a literature search where it was suggested that seropositivity to the antigen was associated with increased odds of >90% transmission-blocking activity (TBA)[282]. Aside from Pfs230, Pfs48/45, Pf47 and NOT1, the other antigens had not been previously studied in the context of TBA, based on *P. falciparum*-derived antigens, at the time of antigen selection. However, TBA following rodent immunisation with *P. berghei* orthologs of PSOP12 and PSOP25 had been described [302,303,517]. Additionally, gene knockout experiments in a rodent malaria model have demonstrated a possible impact on sexual stage development for PSOP1[518,519], TLP[518,519], LAP5[520], G377[521] and MDV1[522,523]. Of the 24 identified proteins, seven of the proteins were conserved *Plasmodium* proteins (denoted as CPPs) of unknown function (**Table 4.1**).

Protein sequences corresponding to each of the 25 antigens were obtained from PlasmoDB and based on the 3D7 sequence. As the full genome of a field isolate from Kilifi was available (PfKE04), I used the annotated genome to obtain sequences corresponding to my GOIs. A comparison of 3D7-based and PfKE04-based isolates by pairwise alignment using Geneious bioinformatics software (version 11.1.2), revealed sequence differences in the genes APP, CPP3, CVMPPP, G377, GEXP01, P47, Pfs230, PHISTa, PIESP15, PSOP25 and TLP, ranging from insertions/deletions to non-synonymous single nucleotide polymorphisms (SNPs) (**9.7 Appendix 7**). For these candidate antigens, I aimed to produce both the 3D7 and PfKE04 version as recombinant protein. I did this to test for differences in immune responses (**Chapter 5**) or functional activity (**Chapter 6**) potentially attributable to the sequence variation. Additionally, owing to the large size of the proteins G377 (PF3D7_1250100) and NOT1 (PF3D7_1103800), two domains were identified and prioritised for analysis.

For G377, the domains chosen for analysis were derived from a study by Alano *et al*. (1995) who were able to express proteins from these domains for further analysis[524] successfully. For NOT1, two short regions were selected to correspond to regions containing predicted B-cell epitopes (predicted using the Immune Epitope Database and Analysis Resource online tool, version 2.6 https://www.iedb.org/). The total number of proteins (including variants) for recombinant production stood at 38 (corresponding to 25 antigens).

Table 4.1: List of gametocyte candidate antigens for protein expression and production

Antigen Name	Gene ID	Molecular weight (kDa)	Protein length (amino acids)	SP	GPI anchor	TM domain	PPED	Rodent malaria knock-out phenotype[a]	AP	Amino acids included in construct [b]	Sequence variation present?[b]
Pfs230[c]	PF3D7_0209000	360	3135	Yes	No	No	No	Male gametes fail to attach to and penetrate female gamete (PBANKA_0306100)	78%	443 – 1132	Yes. 3-amino acid deletion (522-524); SNPs (S163G, V213E, N219K)
Pfs48/45	PF3D7_1346700	46	448	Yes	Yes	Yes	No	Male gametes fail to attach to and penetrate female gamete (PBANKA_1359600)	97.40%	28 – 427	No
VATPase	PF3D7_1354400	19	181	Yes	No	Yes	No	N/A	97.80%	37 – 181	No
PSOP12	PF3D7_0513700	87	735	Yes	No	No	No	No clear phenotype described (PBANKA_1113400)	95.40%	24 – 735	No
CPP1[d]	PF3D7_1118900	29	248	Yes	No	No	No	N/A	94.40%	24 – 248	No
PSOP1	PF3D7_0721700	53	467	Yes	No	No	No	No clear phenotype described (PBANKA_0619200)	90.60%	17 – 467	No
CPP2[d]	PF3D7_0513000	31	269	Yes	No	No	No	N/A	86.20%	20 – 269	No
CVMPPP	PF3D7_1314500	24	206	Yes	Yes	Yes	No	N/A	73.90%	25 – 173	Yes. SNP (P41A)
GEXP01	PF3D7_1253000	56	469	Yes	No	Possibly	Yes	N/A	64.40%	19 – 469	Yes. SNPs (R324C, S391A, E392T, V446I)
P47	PF3D7_1346800	51	439	Yes	Yes	Yes	No	Female gamete fertility affected, reduced number of ookinetes (PBANKA_1359700)	62%	27 – 415	Yes. SNP (P194H)
PHISTa	PF3D7_0115100	35	293	Yes	No	Yes	Yes	N/A	44.90%	25 – 293	Yes. SNP (F113L)
G377[c]	PF3D7_1250100	377	3119	Yes	Yes	No	No		44.70%	184 – 385	No

Antigen Name	Gene ID	Molecular weight (kDa)	Protein length (amino acids)	SP	GPI anchor	TM domain	PPED	Rodent malaria knock-out phenotype[a]	AP	Amino acids included in construct [b]	Sequence variation present?[b]
								Female gametes egress less efficiently from RBCs (PBANKA_1463000)		666 – 1146	Yes. 9-amino acid insertion (103-111); SNP (F421L)
PIESP15	PF3D7_0103900	68	575	Yes	No	Yes	No	N/A	36.60%	30 – 575	Yes. 2-amino acid deletion (221-222)
CPP3[d]	PF3D7_1105800	31	266	Yes	No	Yes	No	N/A	36.10%	19 – 266	Yes. 33-amino acid deletion (11-43)
PSOP25	PF3D7_0620000	60	505	Yes	Yes	Yes	No	Reduction in oocyst numbers (PBANKA_1119200)	26.40%	25 – 475	Yes. 50-amino acid insertion (191-240); SNP (S267L)
CPP4[d]	PF3D7_0208800	28	235	Yes	No	Yes	No	N/A	21.60%	27 – 235	No
CPP5[d]	PF3D7_0309100	21	178	Yes	No	No	No	N/A	12.30%	26 – 178	No
MDV1	PF3D7_1216500	26	221	Yes	No	No	No	Lower number of gametes, reduced ookinete and oocyst production (PBANKA_1432200)	9.30%	23 – 221	No
CPP6	PF3D7_1251000	22	186	Yes	No	Possibly	No	N/A	5.30%	21 – 186	No
NOT1[c, e]	PF3D7_1103800	392	3371	No	No	Yes	No	N/A	4.80%	361 – 409	No
										901 – 1019	No
CPP7[d]	PF3D7_0417000	33	275	Yes	No	Yes	No	No	87%	24 – 275	No
LAP5	PF3D7_1451600	100	865	Yes	No	No	No	Yes. Defective oocysts, impaired sporozoite formation (PBANKA_1315300)	75%	21 – 865	No
APP	PF3D7_1454400	90	777	Yes	No	Yes	No	No	61%	18 – 777	Yes. 1-amino acid deletion (61)
TLP	PF3D7_0616500	160	1371	Yes	No	Yes	No	Yes. Not different from wild type (PBANKA_1116000)	28%	24 – 1371	Yes. 1-amino acid deletion (177), 4-amino acid deletion (982-985); SNPs (K401R, E556K, E557D, Y590D, K591M,

Antigen Name	Gene ID	Molecular weight (kDa)	Protein length (amino acids)	SP	GPI anchor	TM domain	PPED	Rodent malaria knock-out phenotype[a]	AP	Amino acids included in construct [b]	Sequence variation present?[b]
											E592K, E593K, S803C, Y885H)
PEB-P	PF3D7_0303900	23	197	Yes	No	No	No	No	92%	23 – 197	N

ID – identifier; GPI – glycosylphosphatidylinositol, SP – signal peptide, TM – transmembrane domain, PPED – predicted protein export domains, AP – antigenicity percentile, N/A – not available

[a] Rodent malaria gene identified associated with knock-out phenotype provided in brackets.
[b] Amino acid sequence based on the 3D7 variant.
[c] Domains/fragments of these antigens produced due to the size of the protein. For Pfs230 the domain termed 'region C' was chosen, for G377 two domains 'A2 (amino acids 184 – 385)' and 'B (amino acids 666 – 1146)' were chosen and for NOT1 two domains 'domain of unknown function – DUF (amino acids 361 – 409)' and 'Complex (amino acids 901 – 1019)'.
[d] Proteins assigned name CPP to denote conserved *Plasmodium* protein.
[e] Included in the list as responses to the protein correlated with TBA in serum samples from several malaria-endemic regions (Stone *et al.* (2015)[499]).

4.5.1.2 Wheat germ cell-free System protein expression

4.5.1.2.(a) Construct design

Gene sequences corresponding to each of the identified antigens were obtained from PlasmoDB, based on the lab isolate 3D7, and from the field isolate PfKE04. Endogenous nucleotide sequences corresponding to signal peptides, GPI anchors, and transmembrane domains were removed. The resulting sequences were then amplified from either 3D7 genomic DNA, both 3D7 and PfKE04 genomic DNA where sequence variation was present, or cDNA where the genes spanned multiple introns. The PCR products were then run on an agarose gel to verify that each product migrated at the expected size before cloning (**Figure 4.4A**). All but one antigen, PIEPS15, were amplified using the designed primers. Attempts to optimise PCR conditions by gradient PCR in order to test a range of annealing temperatures proved unsuccessful. Therefore, work with PIEPS15 did not proceed further. The nucleotide sequence of PIEPS15 contains several homopolymeric asparagine repeats, a relatively common feature in *P. falciparum* genes,[482] and this could have posed a challenge for successful PCR amplification.

Twenty-four antigens, corresponding to 36 proteins, were thus sub-cloned into the Zero Blunt™ TOPO™ vector, which efficiently ligates blunt-ended PCR products generated during amplification using the Phusion enzyme (**Figure 4.4**). One Shot™ Top10 *E. coli* cells were transformed with the generated constructs and colonies screened the next day to identify constructs that had the required antigen inserts within the cloning site. For those constructs that contained the GOI, the respective gene was then excised from the Zero Blunt™ vector via restriction digestion and ligated into the linearised expression vector, pEU-MCS-E01. Cloning was unsuccessful for 5 of the antigens (APP, GEXP01, PSOP12, Pfs230-C and TLP) as well as one of the domains selected for NOT1 (domain of unknown function (DUF)). I attempted to screen more colonies during colony PCR and tried out a different DNA ligation enzyme. However, none of these approaches worked, and therefore these antigens did not proceed further.

Inability to clone APP, Pfs230-C, TLP and PSOP12 could be explained by their relatively large size (> 2kb) which would have resulted in larger recombinant constructs than could be reliably cloned in *E. coli* cells[525]. Alternatively, factors such

as solvent and salt carry-over from PCR product purification and structural heterogeneity of PCR products can adversely affect ligation and cloning efficiency[526]. Such variables are, however, difficult to detect and monitor during the cloning process. I did, however, attempt to re-clone each failed reaction in a minimum of two independent experiments using freshly purified PCR product and low-salt elution buffers before excluding the antigen from the production pipeline. Further optimisation using a range of insert: vector ratios and ligation conditions may have helped; however, a decision had to be made based on time constraints on what protein constructs to take forward.

Once the expression constructs were successfully generated in the pEU-MCS-E01 plasmid (**Figure 4.4B**), the plasmids were linearised and sequenced using the Illumina MiSeq platform. All reads passed the quality check (had a Phred score of >20 (indicative of approximately 99% accurate base calling[527]) for each base along the entire read length of approximately 200 bp). Hundreds of thousands of reads were generated per plasmid, with tens of thousands of reads mapping back to the reference sequence. This allowed each base to be identified with a higher degree of confidence and allowed the detection of possible in individual reads. Additionally, the trimming of low-quality reads and the use of stringent read-mapping parameters minimised the inclusion of reads with low quality scores in the mapping process, which ensured success in consensus sequence generation. Results from the sequenced plasmids are provided in **9.7Appendix 7**. Mapping of the sequenced reads back to the reference sequence was carried out to verify that the correct insert had been cloned in the right orientation and no mutations had been introduced during the cloning process. Only one gene corresponding to one of the G377 domains (A2) did not match its reference sequence and was excluded from further analysis. A total of 19 antigens (25 proteins) were successfully cloned into the expression plasmid (**Figure 4.5**) for use in protein expression experiments.

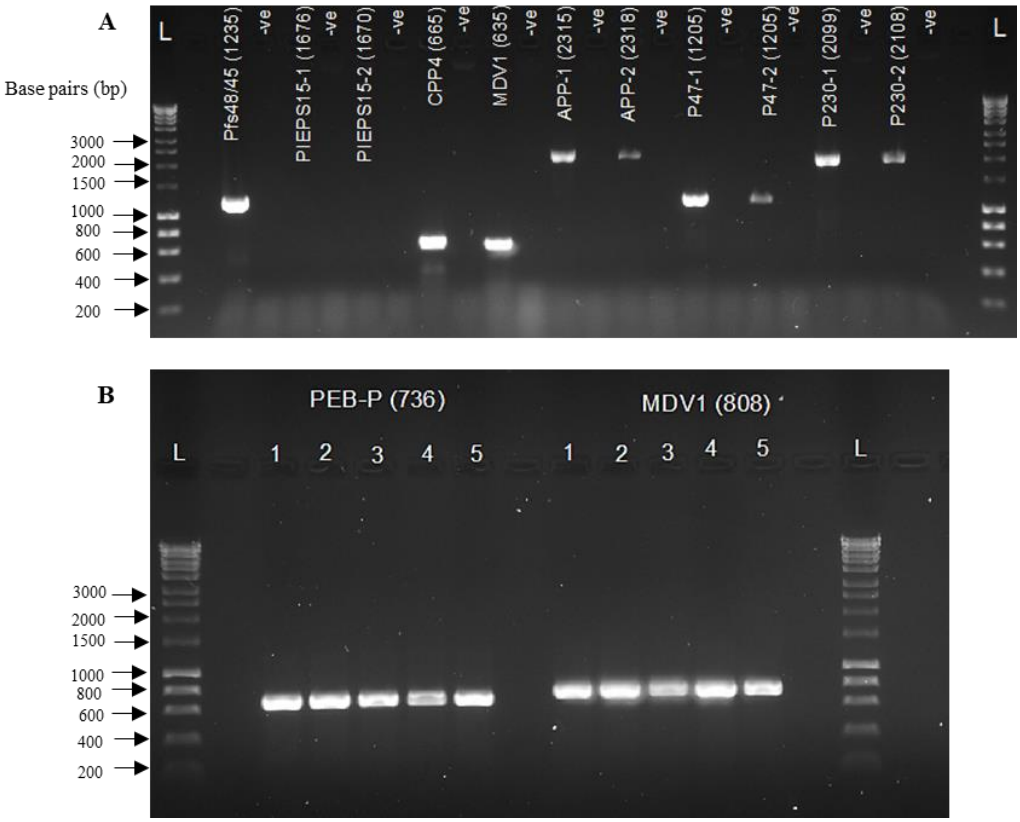


Figure 4.4: Generation of constructs for expression from the identified gametocyte antigens. (A) Specific amplification of a subset of the gametocyte antigens from *P. falciparum* DNA. 1 – amplification of the PfKE04 variant, 2 – amplification of the 3D7 variant. (B) Amplification of the genes of interest from the pEU-MCS-E01 wheat germ expression vector following colony PCR using flank-to-flank primers, the PCR product generated is larger than the original product size for each antigen by 173 base pairs. The numbers 1 - 5 indicate separate colonies screened for integration of the gene of interest into the recombinant plasmid.
-VE – no template control reaction.
Numbers in brackets indicate the expected product size in base pairs.

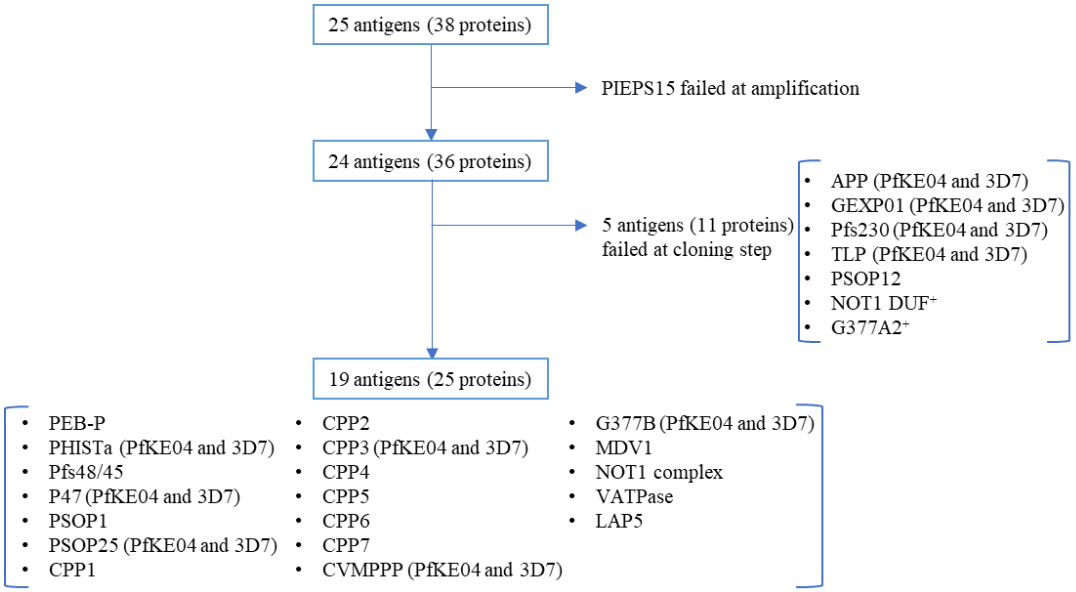


Figure 4.5: Flow diagram illustrating the cloning process for the gametocyte antigens in the wheat germ cell-free system. The diagram shows the protein production pipeline with a description of the antigens that progressed through each cloning step for ultimate protein expression and those that did not.
+ Denotes proteins for which one variant dropped off but another remained in the production pipeline.

4.5.1.2.(b) Protein expression

Small-scale reactions trial experiments were set-up to test the expression of the 19 antigens using the wheat germ cell-free expression kit (WEPRO® 7240 kit) optimised for the purification of histidine-tagged proteins. From the trial experiments, there was evidence of expression for the antigens CPP5, MDV1, CPP3 (PfKE04 and 3D7 variants), PSOP25 (PfKE04 and 3D7 variants), G377B (PfKE04 and 3D7 variants), CPP4 and PEB-P (**Figure 4.6**) at a moderate to high yield (based on the protein band intensity after Coomassie staining). Faint protein bands at the predicted molecular weight were observed for PHISTa (PfKE04 and 3D7), Pfs48/45, PSOP1, CPP1, CPP2, CPP6 and CPP7 indicating low-level protein expression. Initially, co-purification of kit-based wheat germ proteins from the translation mix with the proteins of interest, particularly at 80 kDa, between 48 – 56 kDa and at <10 kDa, was observed. For this reason, I switched to a different protein expression kit optimised for the purification of histidine-tagged proteins (WEPRO® 7240H), utilised a higher concentration of imidazole during the wash steps of protein purification and reduced the quantity of nickel resin used and incubation time during the binding step. This significantly improved protein purity (**Figure 4.7**), and hence the optimised protocol was used for all subsequent purification steps. For G377B, the expressed protein

appeared to migrate at about twice the predicted molecular weight (band appeared at above 100 kDa as opposed to 59 kDa (**Figure 4.6**)). Moreover, the 3D7 version migrated at a slightly higher molecular weight than the PfKE04 version contrary to what was expected. The PfKE04 variant contains a 9-base pair insertion.

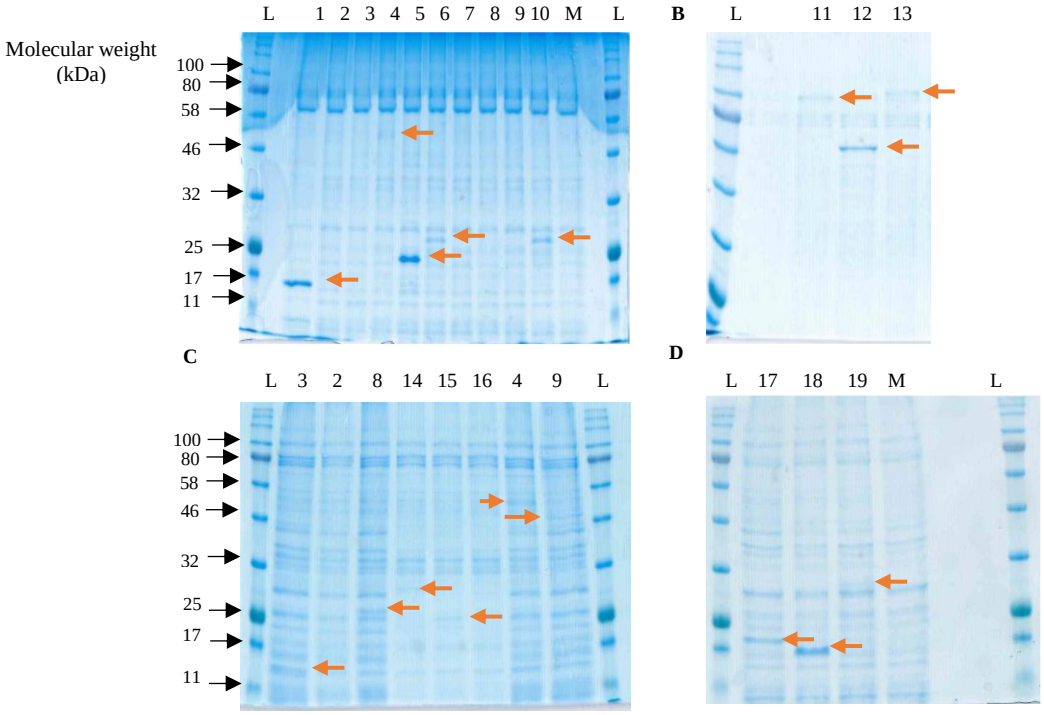


Figure 4.6: Small-scale expression trial using the WEPRO® 7240 wheat germ cell-free expression system. SDS PAGE gels showing purified protein from a subset of the 19 gametocyte antigens that were successfully cloned into the expression plasmid. Orange arrows indicate where evidence of protein expression was observed. (A). 1 – CPP5, 2 – VATPase, 3 – CPP6, 4 – Pfs48/45, 5 – MDVI, 6 – CPP3, 7 – CVMPPP PfKE04, 8 – CPP2, 9 –PSOP1, 10 – CPP3 3D7, M – MOCK; (B). 11 – G377 B PfKE04, 12 – PSOP25 PfKE04, 13 – G377 B – 3D7; (C). 3 – CPP6, 2 – VATPase, 8 – CPP2, 14 – CPP7, 15 – CPP1, 16 – NOT1, 4 – Pfs48/45, 9 – PSOP1; (D). 17 – CPP4, 18 – PEB-P, 19 – PHISTa PfKE04, and M – MOCK.
*L – pre-stained protein ladder

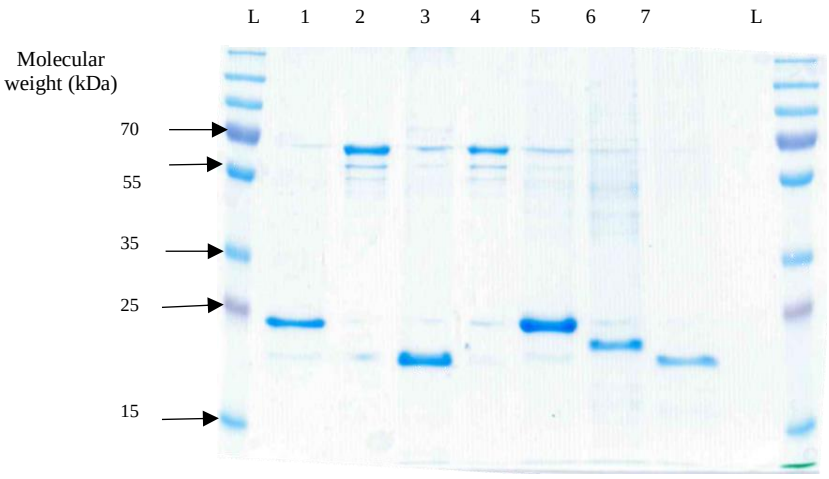


Figure 4.7: Mid-scale expression using the WEPRO® 7240H wheat germ cell-free expression system. SDS PAGE gels showing a subset of proteins purified using an optimised expression kit and more stringent purification protocol. 1 – MDV1, 2 – PSOP25 PfKE04, 3 – PEB-P, 4 – PSOP25 3D7, 5 – G377 A2, 6 – CPP4 and 7 – CPP5
*L – pre-stained protein ladder

Western blot analysis confirmed expression for 7 of the antigens (CPP5, MDV1, CPP3 (PfKE04 and 3D7 variants), PSOP25 (PfKE04 and 3D7 variants), G377B (PfKE04 and 3D7 variants), CPP4 and PEB-P) (**Figure 4.8**). With MDV1 and PSOP25 PfKE04, smaller bands were observed below the expected band size, likely indicating truncated or partially degraded protein products generated during protein synthesis that co-purified with the protein of interest. For the lower yield antigens, only CPP6, CPP7, PSOP1 and PHISTa were observed on the blots.

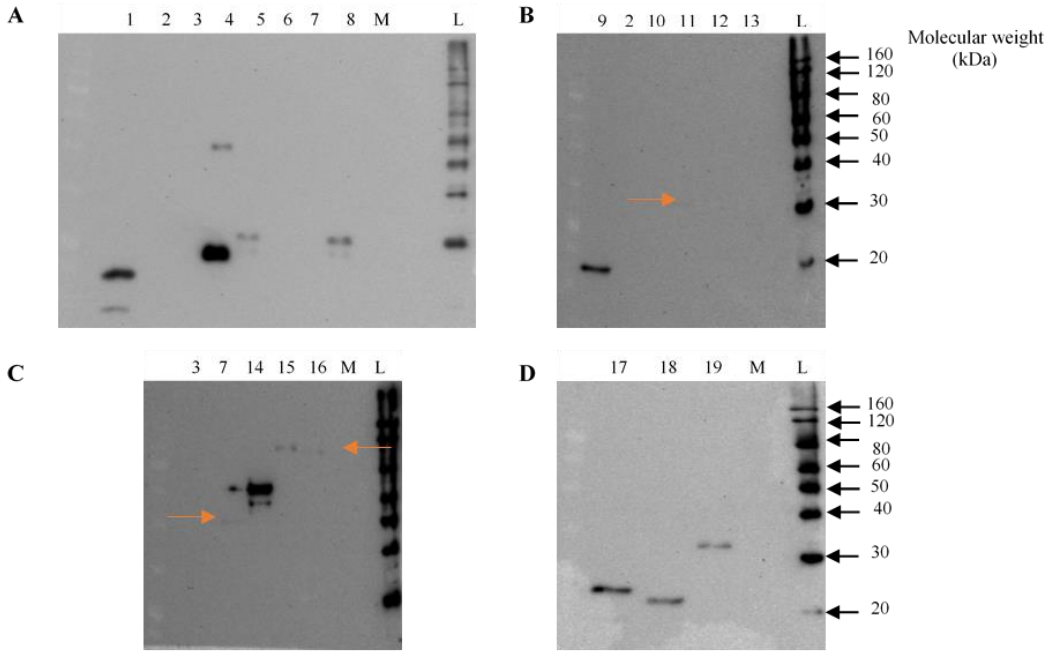


Figure 4.8: Western blot of gametocyte antigens probed with an anti-histidine tag antibody. (A) Shows 8 of the gametocyte antigens. CPP5, MDV1 and CPP3 show up sharply on the blot. (B) Shows the low yield gametocyte antigens with three times as much protein loaded as in (A). CPP6 shows up sharply on the blot while CPP7 shows up faintly. (C) Shows the higher molecular weight proteins, PSOP25 shows up sharply on the blot, with PSOP1 and G377 giving weak signals. (D) Shows CPP4, PEB-P and PHISTa that give clear bands on the blot. 1 – CPP5, 2 – VATPase, 3 – Pfs48/45, 4 – MDV1, 5 – CPP3 PfKE04, 6 – CVMPPP, 7 – PSOP1, 8 – CPP3 3D7, 9 – CPP6, 10 – CPP2, 11 – CPP7, 12 – CPP1, 13 – NOT1 complex, 14 – PSOP25 PfKE04, 15 – G377B 3D7, 16 – G377B PfKE04, 17 – CPP4, 18 – PEB-P, 19 – PHISTa PfKE04 and M – Mock. Faint bands indicated by orange arrows.
*L – pre-stained protein ladder

Plasmids containing the antigens of interest were sent to Dr Eizo Takashima's laboratory (Ehime University, Japan) to validate the results from the small-scale expression trials. There, large scale protein expression using the WGCFS on an automated platform was carried out (**Table 4.2)** to increase the chances of detecting low-yield protein expression. From the expression tests and analysis of protein yields at the 3 ml reaction scale, the antigens CPP4, MDV1, PSOP25 and G377B (PfKE04 and 3D7 variants) were prioritised for the production of a minimum of 1 mg of protein for immunoprofiling and functional assays. Additionally, Pfs230-C and GST (kind gifts from Dr Takashima) were expressed for use as positive and negative controls in further experiments. The domain termed region C of Pfs230 is the target of potent transmission-blocking antibodies[283]. A summary of the protein production process is provided in (**Figure 4.9**).

Table 4.2: Summary of small-scale expression validation at Dr Eizo Takashima's Lab

Antigen	Plasmid Source	Yield (µg/3 ml)	Proceed with large-scale production (Yes/No)
PSOP25 PfKE04	Michelle	174.1	Yes
PSOP25 3D7	Eizo	130.1	Yes
G377 B PfKE04	Michelle	185.0	Yes
G377 B 3D7	Michelle	124.0	Yes
MDV1	Michelle	287.6	Yes
CPP4	Michelle	170.1	Yes
Pfs230-C	Eizo	96.3	Yes
CPP3 PfKE04	Michelle	33.2	No
CPP3 3D7	Michelle	33.2	No
CPP2	Michelle	34.1	No
CPP6	Michelle	39.3	No
CPP7	Michelle	Too low	No
PHISTa PfKE04	Michelle	7.9	No
PHISTa 3D7	Michelle	Too low	No
P47 PfKE04	Michelle	13.6	No
P47 3D7	Michelle	19.6	No
NOT1 complex	Michelle	10.5	No
VATPase	Michelle	40.2	No
CPP1	Eizo	57.6	No
PSOP1	Eizo	27.1	No
CVMPPP	Eizo	27.0	No
LAP5	Eizo	20.2	No
CPP5	Eizo	Too low	No
PSOP12	Eizo	Too low	No

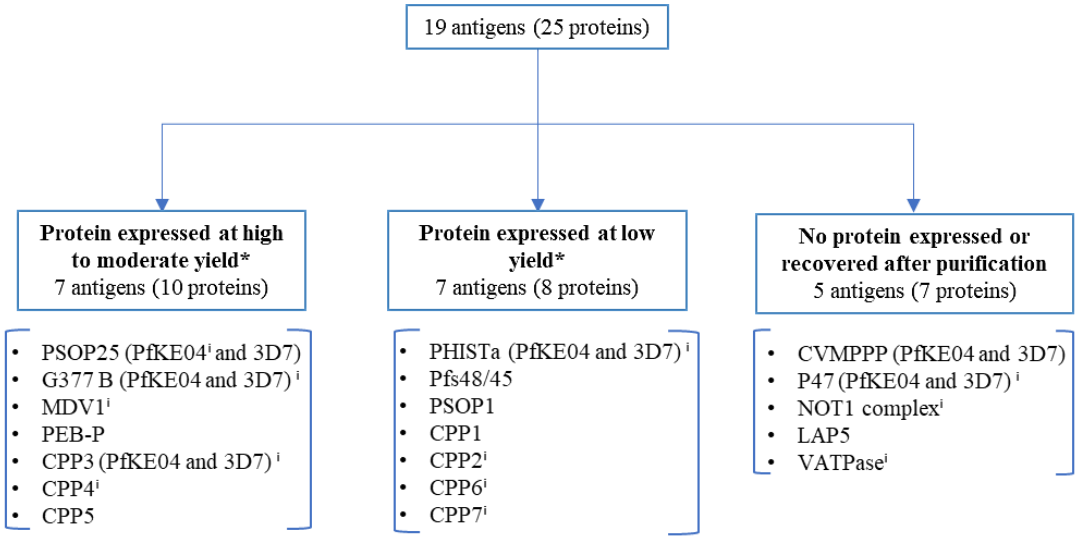


Figure 4.9: Flow diagram illustrating the protein production process for the gametocyte antigens in the wheat germ cell-free system. The diagram shows the protein production pipeline with a description of the antigens that were successfully produced as recombinant protein.
[i] proteins whose corresponding plasmids were sent to Dr Takashima's lab for expression trials.
*yield as determined by protein band intensity after Coomassie staining.

4.5.1.3 Mammalian expression system - HEK293E expression platform

4.5.1.3.(a) Construct design

The HEK293E system was evaluated for the production of a subset of antigens that could not be cloned into the pEU-MCS-E01 vector or whose attempted expression in the WGCFS resulted in low or no protein yield. A total of 15 antigens were selected for protein expression trial experiments. For variant proteins, the PfKE04-based sequence was prioritised for construct design owing to the costs associated with obtaining commercial constructs. Endogenous signal peptide sequences were replaced by the tPA signal sequence for enhanced protein expression[508]. Additionally, a Kozak sequence was introduced upstream of the ATG start codon[515], internal glycosylation sites were modified and the nucleotide sequences optimised for human codon usage to further enhance expression[485].

As with the sequences for wheat germ expression, GPI anchors and transmembrane domains were excluded from the final sequence used to design constructs for expression. The genes of interest were amplified from the provided GeneArt®-provided construct (**Figure 4.10A**) and cloned into the destination pOPINGS vector

via in-fusion cloning. Colony PCR was then used to verify that the inserts were successfully incorporated into the pOPINGS vector (**Figure 4.10B**). Cloning of PIEPS15 and PSOP12, into the pOPINGS vector was unsuccessful, and therefore protein expression was attempted for 13 of the antigens. These two antigens were also unsuccessful in the wheat germ system. This suggests that they would require further optimisation to test out a range of cloning conditions and vectors, or the expression of domains of these genes. A summary of the cloning process is provided in **Figure 4.11**.

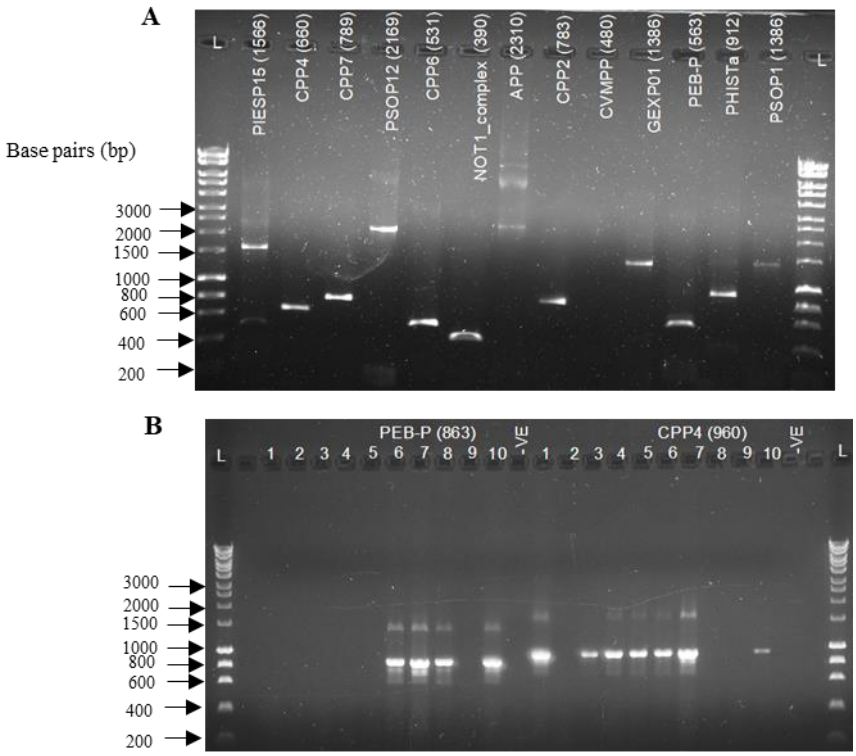


Figure 4.10: Generation of constructs for the expression of the identified gametocyte antigens in the HEK293E mammalian system. (A) Specific amplification of a subset of the gametocyte antigens from GeneArt® provided constructs. (B) Amplification of the genes of interest from the pOPINGS expression vector following colony PCR using T7 forward sequencing primer and an antigen-specific reverse primer. The primer combination generates a PCR product that is larger than the original PCR product by 300 base pairs. The numbers 1 - 10 indicate separate colonies screened for integration of the gene of interest into the recombinant plasmid.
-VE – no template control reaction to test for non-specific amplification.
Numbers in brackets indicate the expected size of the PCR product in base pairs.

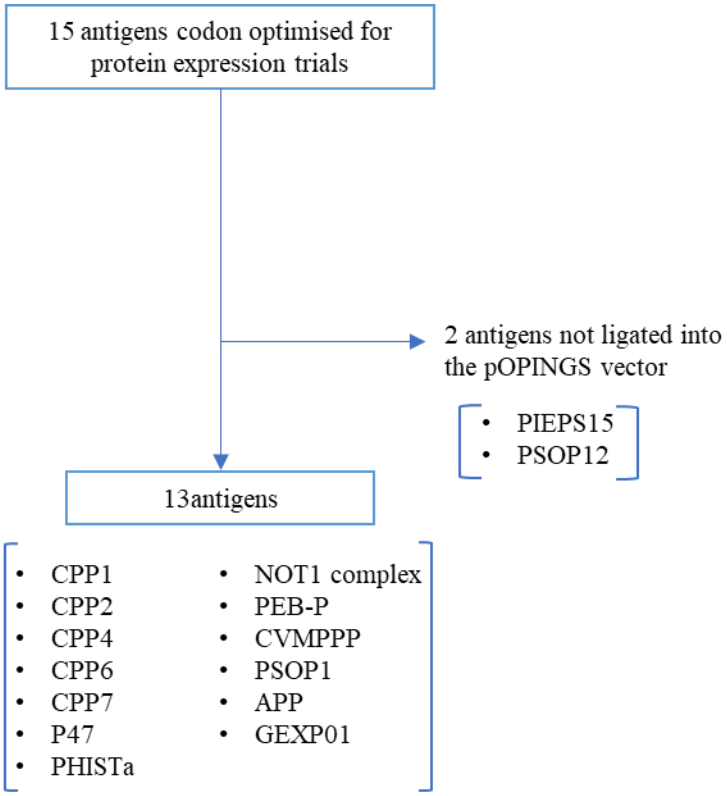


Figure 4.11: Flow diagram illustrating the cloning process for the gametocyte antigens in the mammalian expression system. The diagram shows the protein production pipeline with a description of the antigens that progressed through each cloning step for ultimate protein expression.

4.5.1.3.(b) Protein expression

Of the 13 antigens, three were expressed at a high yield, two at a low yield, and for eight, no evidence of protein expression was observed (**Figure 4.12**). The yield was assessed based on the resulting band intensity after Coomassie staining. A single 20 ml expression trial experiment was carried out as an initial screen for protein expression without further optimisation tested due to time constraints. PEB-P, CVMPPP and PSOP1 protein expression were confirmed by western blot analysis using an antibody against the histidine tag. (**Figure 4.13**) Therefore, they proceeded to large-scale production. Additionally, human secreted alkaline protease (SEAP) was also expressed to serve as a negative control in further assays. A summary of protein production in the mammalian expression system is provided in **Figure 4.14**.

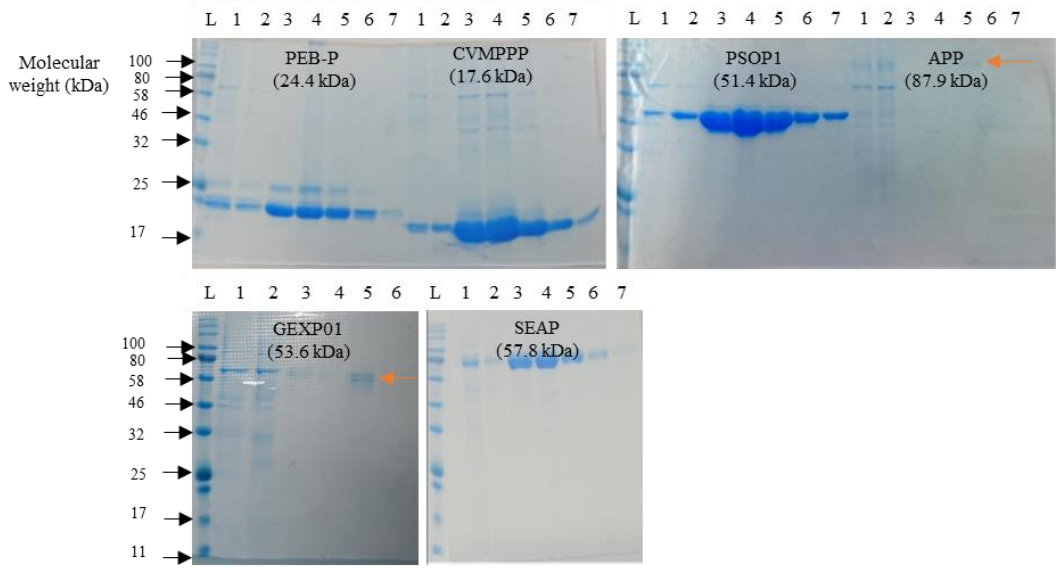


Figure 4.12: Small-scale expression trial using the HEK293E mammalian expression system. SDS PAGE gels showing purified protein from a subset of the gametocyte antigens where evidence of protein expression was observed. Orange arrows indicate faint protein bands.
*L – pre-stained protein ladder
1 – flow-through, 2 – wash 1, 3 – elution 1, 4 – elution 2, 5 – elution 3, 6 – elution 4, 7 – elution 5

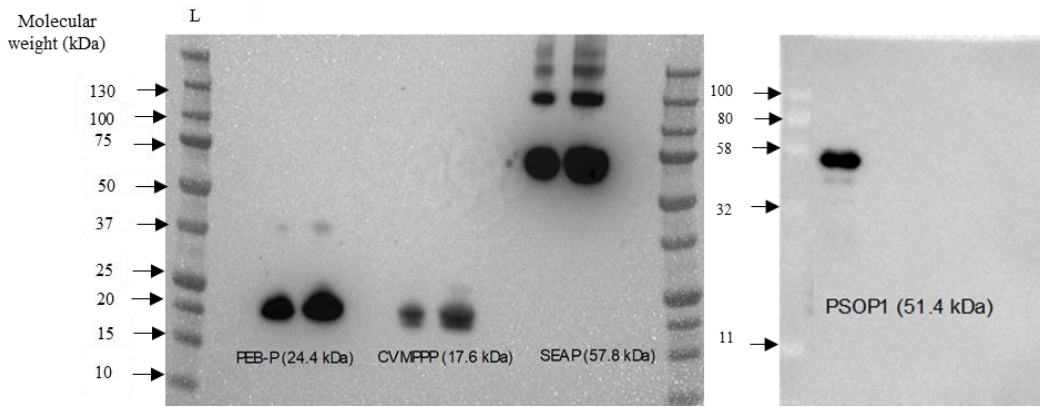


Figure 4.13: Western blot of gametocyte antigens probed with anti-histidine tag antibody. The expressed CVMPPP, PEB-P, PSOP1 and SEAP show up sharply on the blot and migrate at the expected size. Numbers in brackets indicate the expected molecular weight in kilodaltons (kDa). A histidine-tagged protein ladder was included for sizing of the fragments.

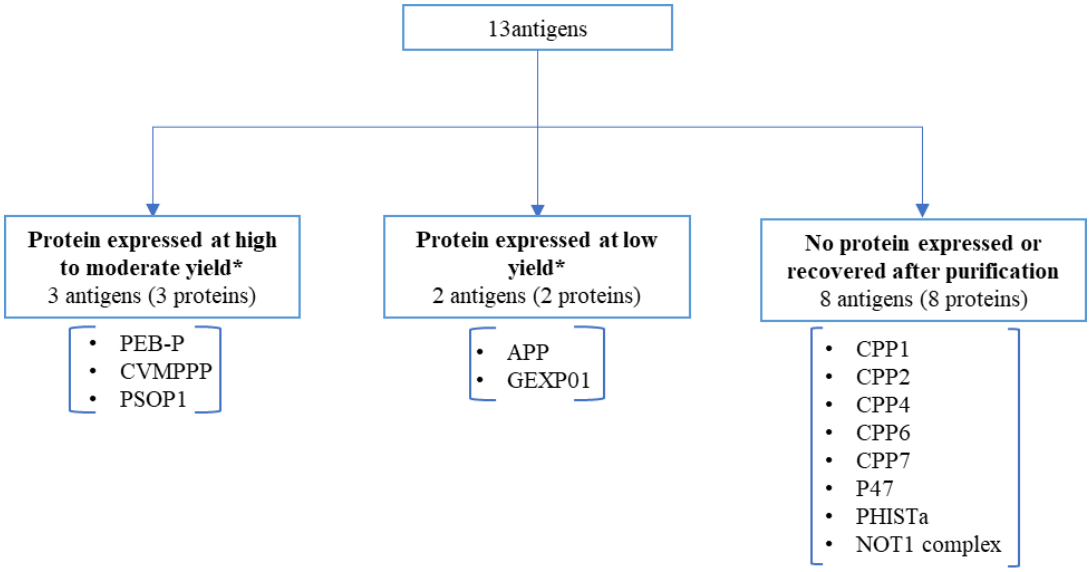


Figure 4.14: Flow diagram illustrating the protein production process for the gametocyte antigens in the mammalian expression system. The diagram shows the protein production pipeline with a description of the antigens that were successfully produced as recombinant protein.
*yield as determined by protein band intensity after Coomassie staining.

4.5.1.4 Mass Spectrometry confirmatory analysis (LC/MS/MS)

Mass spectrometry analysis was carried out for 8 of the gametocyte antigens that had been successfully expressed at high yield (CPP4, MDV1, PEB-P, PSOP25 PfKE04, G377B PfKE04 and 3D7, CVMPPP and PSOP1) to confirm the identity of the protein. The LC/MS/MS analysis was carried out in-house at KWTRP. Peptides generated from each of the purified proteins were blasted against a database of the *P. falciparum* proteome for identification. For each antigen tested, the peptide sequences generated positively matched the respective target protein.

G377B appeared to migrate much slower on the gel than expected, with the 3D7 variant appearing to migrate slower than the PfKE04 variant (**4.5.1.2.(b)**). This was contrary to what was expected. The PfKE04 variant is predicted to contain an extra copy of the 'PLNHEEDNF' motif (three in contrast to two copies in the 3D7 variant). Moreover, a non-synonymous SNP (F1077L) is present in PfKE04 further distinguishing the variants. To investigate this discrepancy further, I sent purified protein from both antigens to the Cambridge Centre for Proteomics for LC/MS/MS analysis. From the results of the peptide sequencing (**Figure 4.15**) the PfKE04 variant was shown to contain three copies of the 'PLNHEEDNF' motif and the associated

SNP. On the other hand, none of the generated peptides from the 3D7 variant mapped onto the 'PLNHEEDNF' motif region. Additionally, the 3D7 variant seemed to contain the same SNP as the PfKE04 variant making it difficult to explain the observed discrepancies between the variants definitively.

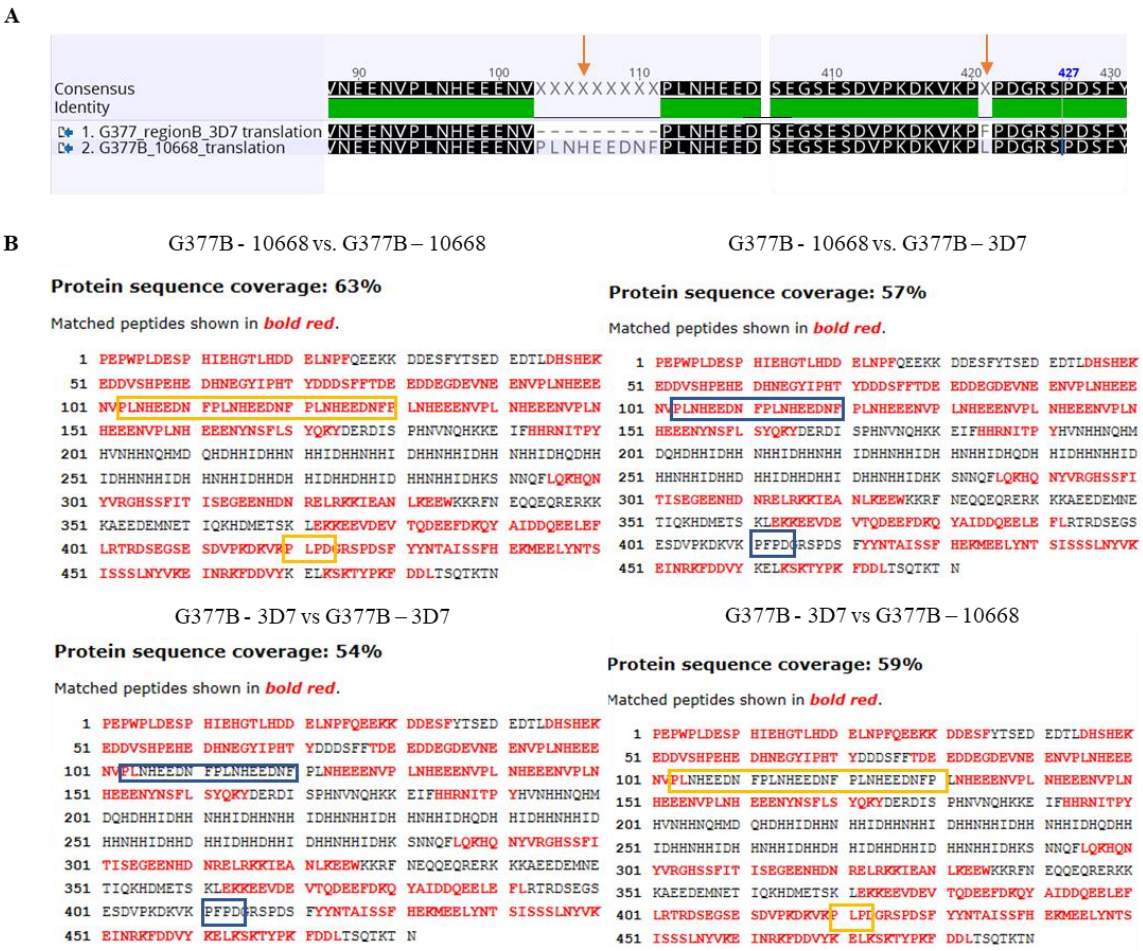


Figure 4.15: G377B variant analysis. (A). Pairwise alignment of protein sequences of G377B PfKE04 and 3D7 variants with red arrows denoting the insertion of the 'PLNHEEDNF' and the (F1077L) SNP. (B) Mapping of peptides from LC/MS/MS analysis of purified G377B protein variants. Orange boxes highlight the regions of variation from the PfKE04 sequence while blue boxes highlight regions from the 3D7 sequence.

4.5.2 Gamete and ookinete antigens

4.5.2.1 Potential antigens identified for analysis

A shortlist of gamete and ookinete antigens was prepared from published gamete and ookinete proteome data[364] as well as from experimentally-identified potentially surface localised proteins (Blagborough A and Angrisano F, personal communication). Proteins from the proteome dataset were searched on pathogen databases to identify genes with signal peptides, transmembrane domains, GPI anchors, protein export motifs, predicted antigenicity (TDR Targets) and for a described knock-out phenotype in rodent malaria models. This was done to generate criteria for antigen down-selection Following the selection process described in **4.4.2.2**, a list of 21 antigens was generated for analysis (**Table 4.3**).

The list included P28 for use as a positive control in the functional assays. The *P. falciparum* ortholog of P28, Pfs25, has been shown to induce antibodies with potent TBA[292,322,323]. Of the antigens identified, SOAP[304], GAP50[528], GEST[74], PH[386] and CHT1[326] have had anti-sera raised against them previously to test for TBA. The antigens SEP1, MFR5, and PLP2 have not been directly assessed for transmission-blocking activity. However, gene knock-out studies have shown that their disruption adversely affects parasite development within the midgut. The other antigens were uncharacterised as TBV targets at the time of selection, with 6 of them being CPPs.

To aid future analysis in a human model of infection, I identified the *P. falciparum* orthologs of these proteins where available and examined possible sequence variation with the field isolate PfKE04. Only 4 of the 21 antigens (GEST, PBCPP1, SOAP and PLP2) had sequence variation that ranged from insertions/deletions to non-synonymous SNPs, indicating a high degree of conservation (**9.7 Appendix 7**).

Table 4.3: List of gamete and ookinete candidate antigens for evaluation as transmission-blocking antigens

Gene Name	Gene ID	*P. falciparum* Ortholog	Molecular Weight (kDa)	Protein length (amino acids)	SP	GPI anchor	TM Domain	RMgmDB data	AP [a]	Amino acids included in construct	Sequence variation present?[b]
P28	PBANKA_0514900	PF3D7_1030900	23.52	213	Yes	Yes	Yes	Yes, slight reduction in the number of ookinetes developing into oocysts.	99%	23 – 193	No
PBCPP1[c]	PBANKA_1105300	PF3D7_0505700	55.058	455	Yes	No	Yes[+]	No	1%	19 – 455	Yes. 26-amino acid deletion (211-236); SNP (D865E)
PBCPP2[c]	PBANKA_1463900	PF3D7_1251000	21.594	183	Yes	No	No	No	5%	20 – 183	No
PBCPP3[c]	PBANKA_1112700	PF3D7_0513000	30.586	268	Yes	No	No	Yes, but no sexual stage phenotype defined.	86%	20 – 268	No
PBCPP4[c]	PBANKA_0719100	PF3D7_0417000	33.51	281	Yes	No	No	No	87%	24 – 281	No
GAP50	PBANKA_0819000	PF3D7_0918000	44.307	395	Yes	No	Yes	No	39%	25 – 383	No
PBfam	PBANKA_1100700	N/A	34.524	296	Yes	No	No	No	N/A	26 – 296	No
SOAP	PBANKA_1037800	PF3D7_1404300	18.2	166	Yes	No	No	Yes, reduced oocyst production.	24%	21 – 166	Yes. SNP (G93R)
SEP1	PBANKA_0524800	PF3D7_1102700	12.234	115	Yes	No	Yes[+]	Yes, but no sexual stage phenotype defined.	89%	23 - 115	No
GEST	PBANKA_1312700	PF3D7_1449000	28.797	249	Yes	No	No	Yes, reduced gamete egress, fertilisation and ookinete-oocyst production.	31%	21 – 249	Yes. SNPs (Q123K, Q141N, N202D)
PH	PBANKA_0417200	PF3D7_0904200	33.011	286	Yes	No	Yes	Yes, reduced oocyst production.	77%	21 – 286	No
CHT1	PBANKA_0800500	PF3D7_1252200	72	648	Yes	No	No	Yes, reduced oocyst production.	83%	19 – 648	No

Gene Name	Gene ID	*P. falciparum* Ortholog	Molecular Weight (kDa)	Protein length (amino acids)	SP	GPI anchor	TM Domain	RMgmDB data	AP [a]	Amino acids included in construct	Sequence variation present?[b]
AP	PBANKA_0813400	PF3D7_0912400	53.18	453	Yes	No	Yes	No	78%	21 – 404	No
PBCPP5[c]	PBANKA_1452300	PF3D7_1237700	23.992	210	Yes	No	Yes	No	1%	24 – 163	No
M1AAP	PBANKA_1410300	PF3D7_1311800	123.351	1064	Yes	No	No	No	84%	19 – 1064	No
PBCPP6[c]	PBANKA_1452500	PF3D7_1237900	81.03	722	Yes	No	No	No	N/A	27 – 722	No
THX	PBANKA_0942500	N/A	49.065	420	Yes	No	No	No	N/A	24 – 420	No
SERCA[d]	PBANKA_0207000	PF3D7_0106300	127.352	1120	No	No	Yes[+]	No	66%	1 – 1120	No
VAMP[d]	PBANKA_1303700	PF3D7_1439800	26.901	234	No	No	No	No	69%	1 – 234	No
MFR5[d]	PBANKA_0918300	PF3D7_1129900	57.089	500	No	No	Yes[+]	Strongly reduced exflagellation, no sporozoites produced.	52%	1 – 500	No
PLP2[d]	PBANKA_1432400	PF3D7_1216700	114.346	999	No	No	No	Male gamete shows abnormal exflagellation, produces only one gamete.	51%	1 – 999	Yes. 6-amino acid deletion (472-477); SNP (K981R)

ID – identifier; GPI – glycosylphosphatidylinositol, SP – signal peptide, TM – transmembrane domain, AP – antigenicity percentile, N/A – not available

[+] Domain structure indicates a multi-pass membrane protein.

[a] Antigenicity predicted for the *P. falciparum* ortholog.

[b] Sequence variation identified by comparing the *P. falciparum* ortholog to the PfKE04 variant.

[c] Proteins assigned name CPP to denote conserved *Plasmodium* protein, prefix PB- added to differentiate from the conserved *P. falciparum* proteins.

[d] Lack a signal peptide though have been localised to the surface by laboratory experiments or prediction by bioinformatic analyses.

4.5.2.2 Wheat germ cell-free system

4.5.2.2.(a) Construct Design

Sequences corresponding to the genes of interest were retrieved from PlasmoDB and were based on the rodent malaria parasite *Plasmodium berghei* ANKA strain. As with the gametocyte antigens (refer to **4.5.1.2**) signal peptide sequences, GPI anchors and TM domains (where possible) were omitted from the gene sequence used for construct design. PBCPP1, SEP, SERCA and MFR5 contained internal TM domains which would have required producing several truncated forms of the protein, and hence the transmembrane domains were left intact. The selected regions of the GOI were then amplified from cDNA prepared from RNA extracted from gamete and ookinete stage *P. berghei* parasites. Again, the PCR primers contained a histidine tag at the end of the protein sequence to allow protein purification by affinity chromatography using nickel resin. For some of the antigens, gradient PCR optimisation was necessary to determine optimal annealing temperatures for amplification as the predicted annealing temperature did not work.

The PCR products were then run on agarose to confirm that they migrated at the expected size (**Figure 4.16A and B**). In order to generate the final constructs for protein expression, the amplified PCR products were sub-cloned into the ZeroBlunt™ vector before being cloned into the pEU-MCS-E01 expression vector as previously described. The final plasmids were screened for the presence of the GOI by colony PCR, also as described (**Figure 4.16C**). For 6 of the antigens (GAP50, PBCPP4, PBCPP1, SERCA, PBCPP6, and PLP2), cloning was unsuccessful despite optimisation attempts as described in **4.5.1.2.(a)**. Therefore, these antigens did not proceed for protein expression trials. As with the gametocyte antigens, antigens greater than 2000 base pairs presented a challenge for cloning with only M1AAP cloned into the pEU-MCS-E01 plasmid. At this point, I opted to focus on the 15 antigens that were cloned successfully for the protein expression trials (**Figure 4.17**).

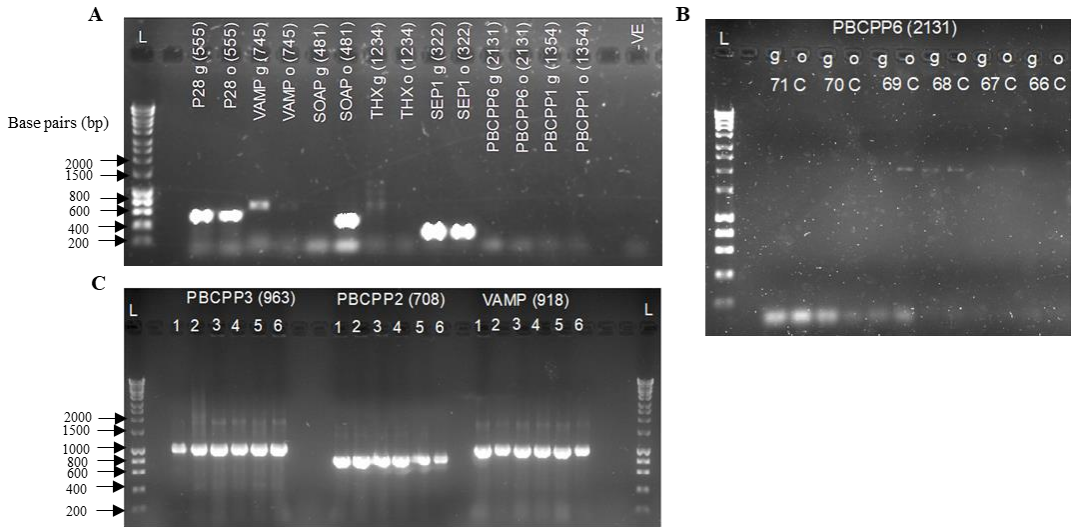


Figure 4.16: Generation of constructs for expression from the identified gamete and ookinete antigens. (A) Specific amplification of a subset of the antigens from *P. berghei* gamete and ookinete DNA. g – gamete cDNA used as the template, o – ookinete cDNA used as the template. (B) Gradient PCR used to optimise PCR conditions for the successful amplification of PBCPP6. (C) Amplification of the genes of interest from the pEU-MCS-E01 wheat germ expression vector following colony PCR using flank-to-flank primers. The numbers 1 - 6 indicate separate colonies screened for integration of the gene of interest into the recombinant plasmid.
-VE – no template control reaction.
* Numbers in brackets indicate the size in base pairs of the expected PCR product.

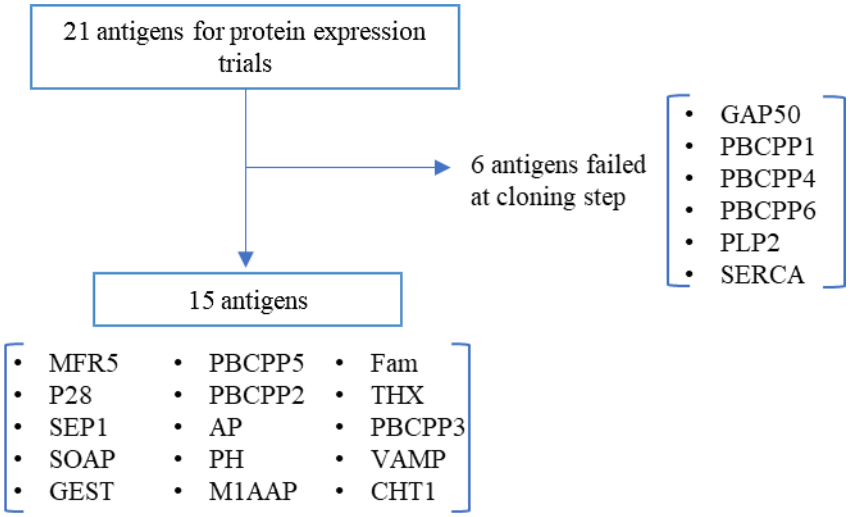


Figure 4.17: Flow diagram illustrating the cloning process for the gamete and ookinete antigens in the wheat germ cell-free expression system. The diagram shows the protein production pipeline with a description of the antigens that progressed through each cloning step for ultimate protein expression and those that did not.

4.5.2.2.(b) Protein expression

Fifteen antigens proceeded to protein expression trials in small-scale reactions using the same protocol set up during the expression of the gametocyte antigens in the wheat germ system (refer to **4.5.1.2**). For 10 of the antigens, there was no evidence of protein expression, with only three antigens showing up on the SDS PAGE gels (**Figure 4.18**). The low expression rate was contrary to what I had observed with the gametocyte antigens (refer to **4.5.1.2**). To confirm these results, I picked 8 of the antigens and loaded three times as much protein onto an SDS PAGE gel prior to carrying out a western blot in a bid to detect lowly expressed proteins. From the blot, expression of Fam was confirmed while PBCPP3 and VAMP showed up faintly on the blot. This suggested low protein yields for at least some of the antigens. Therefore, I sought to explore whether yields could be improved by adding a glutathione-S-transferase (GST) fusion tag to the N-termini of the proteins. GST tagging can aid the production of soluble protein as has been described[529,530].

I first carried out a trial with the THX protein and cloned the PCR product (containing a histidine sequence added by the reverse PCR primer) into the pEU-E01-GST-N2 vector (**9.6 Appendix 6**). This vector contains an N-terminal GST tag sequence (**Figure 4.19A**). I then compared protein expression between the single tag and the dual tag proteins in a small-scale expression trial. Unfortunately, the GST tag did not appear to improve protein yield for THX (**Figure 4.19B**), and therefore I did not attempt this approach for the rest of the antigens. Owing to the low success experienced with expressing the gamete and ookinete proteins in the wheat germ system (**Figure 4.20**), I opted to re-attempt protein expression in the bacterial system. However, as protein expression was observed for CHT1, THX and Fam, these proteins were sent to Dr Takashima's lab for expression validation and possible production scale-up (**Table 4.4**). From the yields obtained after large-scale production, CHT1, THX and Fam were considered for functional assays; however, cost considerations precluded production of Fam.

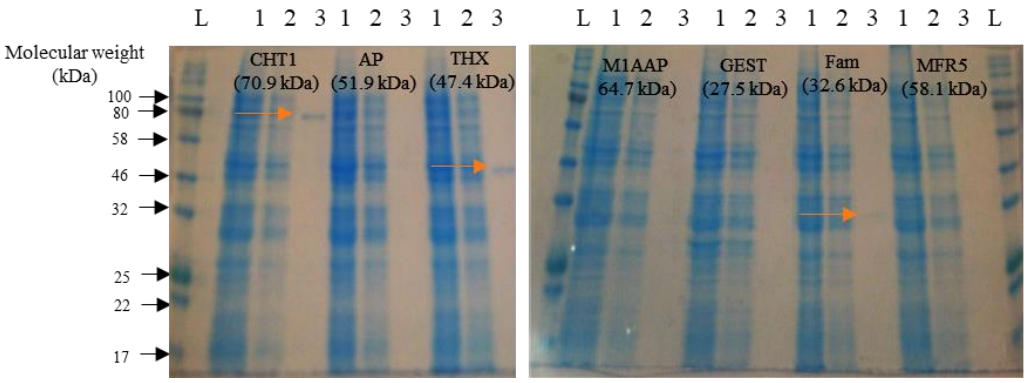


Figure 4.18: Small-scale expression trial using the WEPRO® 7240H wheat germ cell-free expression system. SDS PAGE gels showing purified protein from a subset of the 15 gametocyte antigens that were successfully cloned into the expression plasmid. Orange arrows indicate possible protein expression.
* L-pre-stained protein ladder
1 – flow-through, 2 – wash 1, and 3 - elution

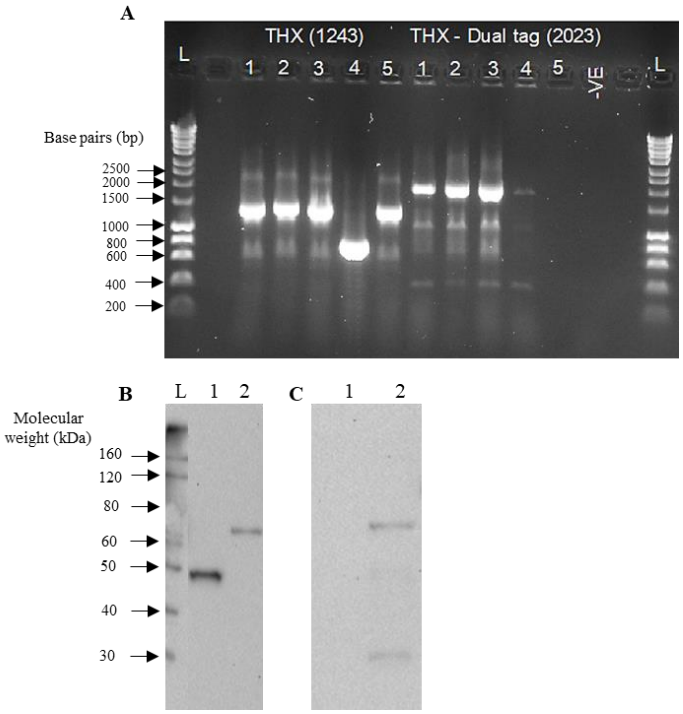


Figure 4.19: Dual tagging of THX protein for improved protein expression. (A) Amplification of the genes of interest from the pEU-MCS-E01 and pEU-E01-GST-N2 (dual tag) wheat germ expression vectors following colony PCR using flank-to-flank primers. The numbers 1 – 5 indicate separate replicate amplifications. (B) Western blots of purified THX protein probed with the anti-histidine tag antibody. 1 – single tagged THX (histidine tag); 2 – dual tagged THX (glutathione-s-transferase (GST) and histidine tags). (C) Western blots of purified THX protein probed with GST tag antibody. 1 – single tagged THX (histidine tag); 2 – dual tagged THX (GST and histidine tags).

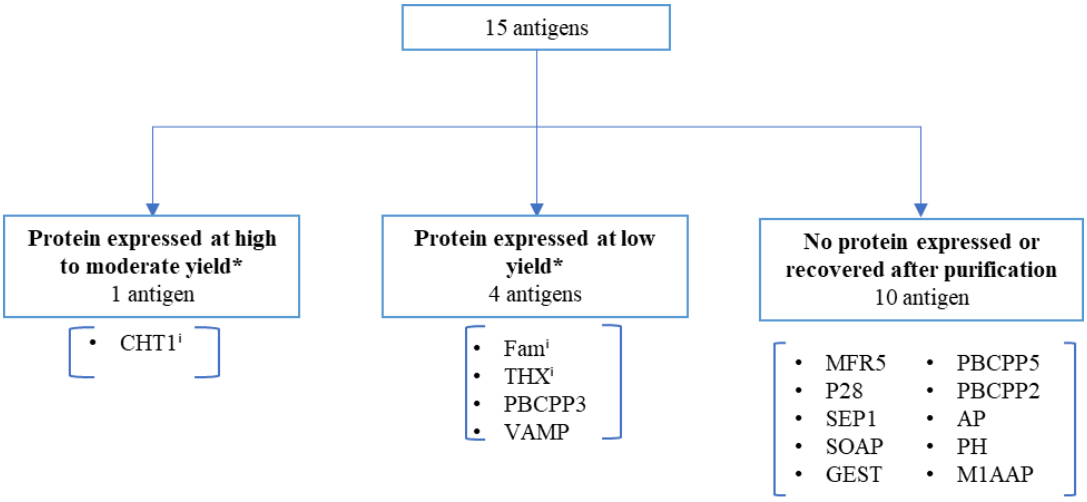


Figure 4.20: Flow diagram illustrating the protein production process for the gamete and ookinete antigens in the wheat germ cell-free system. The diagram shows the protein production pipeline with a description of the antigens successfully produced as recombinant protein.
[i] proteins whose corresponding plasmids were sent to Dr Takashima's lab for expression trials
*yield as determined by protein band intensity after Coomassie staining.

Table 4.4: Summary of small-scale expression validation at Dr Eizo Takashima's Lab

Antigen	Plasmid Source	Yield (µg/3 ml)	Proceed with large-scale production (Yes/No)
CHT1	Michelle	386.46	Yes
THX	Michelle	76.14	Yes
Fam*	Michelle	120.24	Yes

* Owing to cost considerations, Fam was not expressed at a larger scale for functional assays

4.5.2.3 Bacterial expression system

4.5.2.3.(a) Construct design

The same nucleotide sequences corresponding to the genes of interest identified during construct design for expression in the wheat germ system were used to prepare constructs for expression in Rosetta-gami™ bacterial cells. The regions of interest were amplified from parasite genetic material using primers compatible with infusion cloning into the pOPINS3C vector. This vector was chosen as it contains a small ubiquitin-related modifier (SUMO) tag sequence upstream of the gene of interest that fuses with the target protein during expression. The SUMO tag has been described to

increase protein solubility[529]. Additionally, the vector contains an N-terminal histidine tag upstream of the SUMO tag to allow for affinity purification using nickel beads.

Again, cloning was unsuccessful for PLP2, PBCPP1 and SERCA. Additionally, AP was not successfully cloned. Cloning was successful for 17 of the antigens, however, for six of these (PBCPP4, PBCPP6, M1AAP, SEP, THX and GEST), there appeared to be discrepancies in the sequenced plasmids when mapped onto the reference sequence (**9.7 Appendix 7**). The discrepancies indicated that possible mutations had been introduced during the cloning process, at least in the plasmids selected for sequencing. For verification, additional colonies could have been screened, and their plasmids extracted and sequenced. However, owing to time constraints, these were excluded, and hence 11 antigens proceeded to expression trials (**Figure 4.21**).

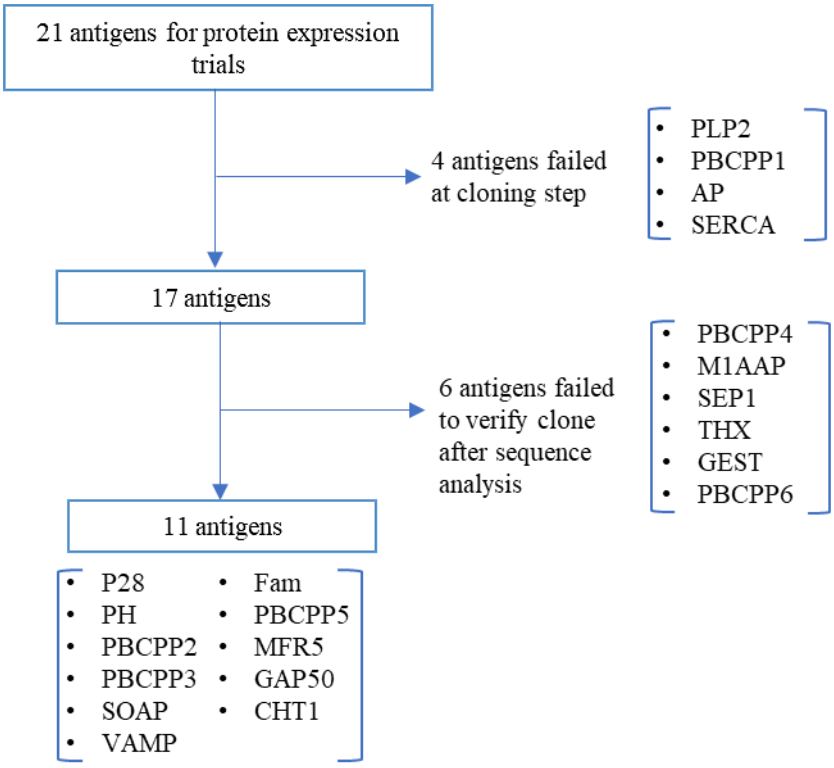


Figure 4.21: Flow diagram illustrating the cloning process for the gamete and ookinete antigens in the Rosetta-gami™ bacterial expression system. The diagram shows the protein production pipeline with a description of the antigens that progressed through each cloning step for ultimate protein expression and those that did not.

4.5.2.3.(b) Protein expression

Small scale expression trials were carried out for the 11 antigens. Expression was observed for all but MFR5 and GAP50 (**Figure 4.22**), giving a much higher success rate than the WGCFS. I also attempted to express SUMO tag protein as a negative control; however, no protein was detected in the expression trial. Furthermore, though CHT1 was expressed, it was not possible to recover soluble protein during protein purification, possibly due to protein aggregation during protein expression leading to the production of insoluble protein[525]. Of the eight remaining antigens, P28, PH, SOAP, VAMP, PBCPP2, PBCPP3 were reasonably expressed and for these, protein production was scaled up for functional work (**Figure 4.23**). A summary of the protein production pipeline for the gamete and ookinete-stage antigens in the bacterial system is provided in **Figure 4.24**.

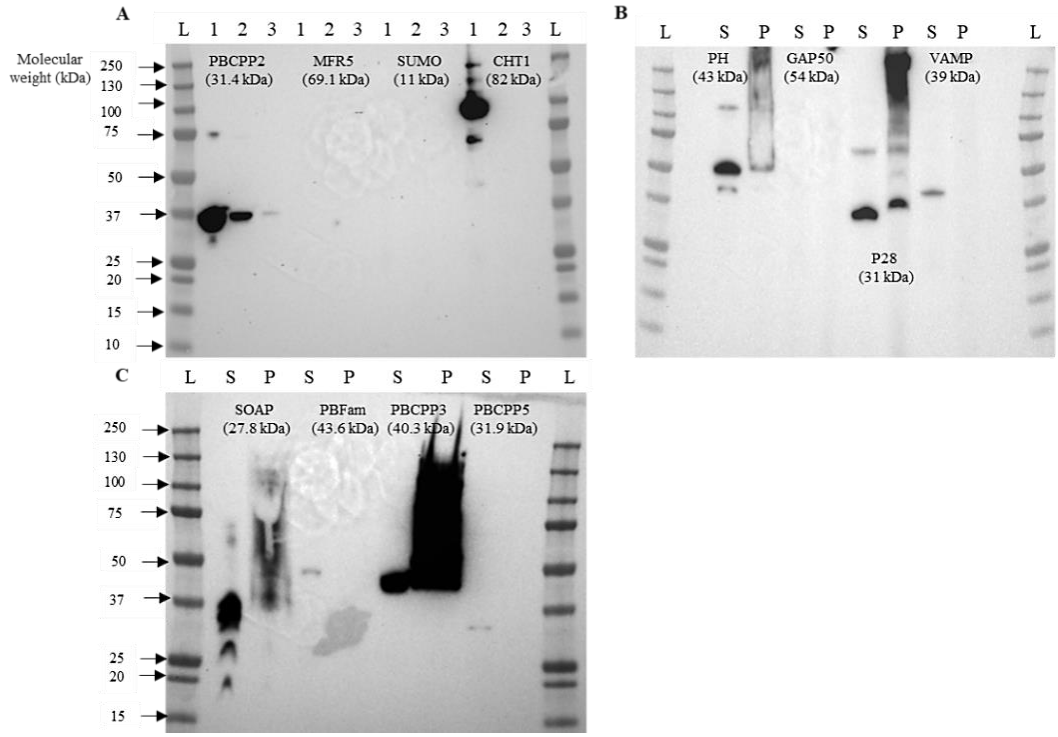


Figure 4.22: Western blot analysis of the small-scale expression trial. Small scale expression trials for 11 of the gamete and ookinete proteins in the Rosetta-gami™ bacterial expression system. Soluble and pellet fractions were analysed to test for evidence of protein expression. For (A) the extracted protein was purified before analysis by western blot, for (B) and (C), un-purified protein extract and the pellet fraction were analysed on the western blot.
L – pre-stained protein ladder
1 – pellet, 2 – flow-through, 3 – eluate. S- soluble fraction and P – pellet fraction.

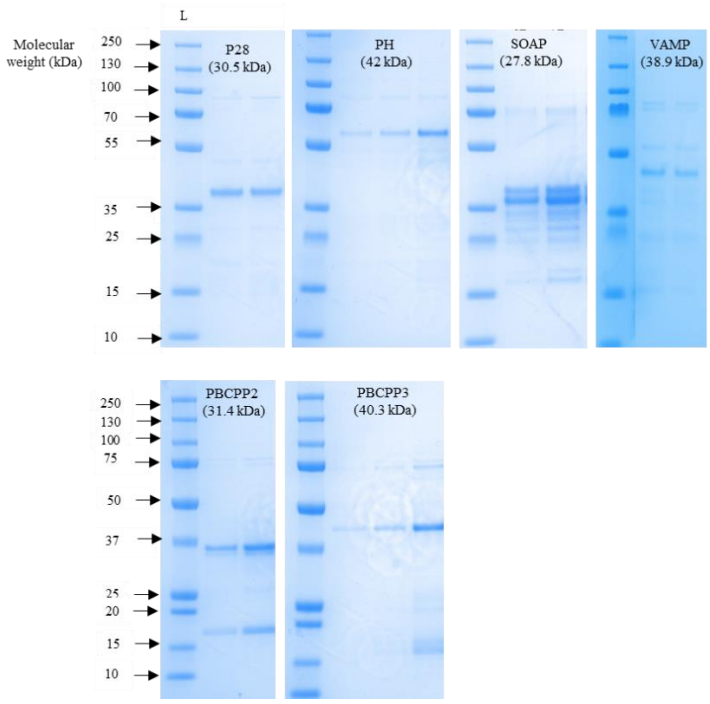


Figure 4.23: Purified protein from the gamete and ookinete antigens after large scale protein production. SDS PAGE gels showing purified protein from six of the gamete and ookinete antigens that were successfully expressed in the bacterial expression system.
*L – pre-stained protein ladder

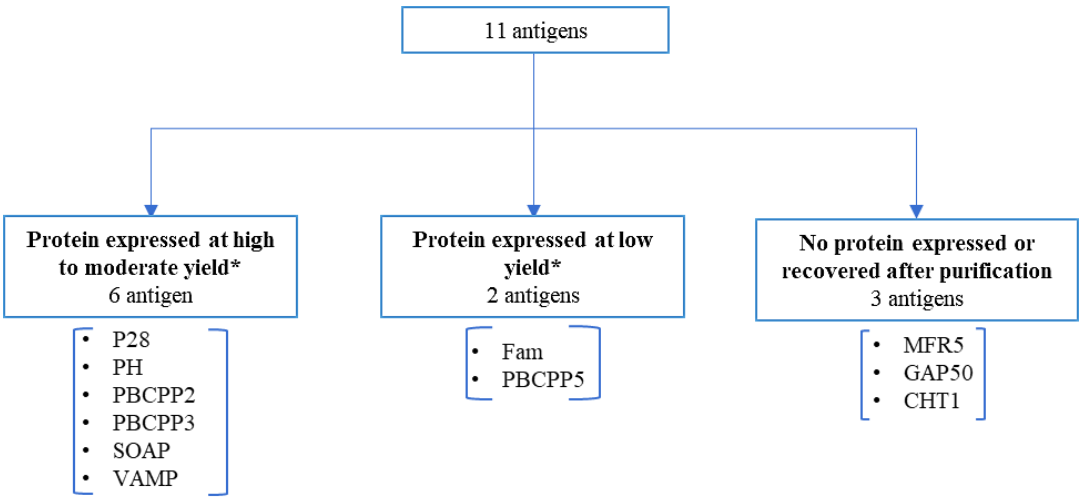


Figure 4.24: Flow diagram illustrating the protein production process for the gamete and ookinete antigens in the Rosetta-gami™ bacterial expression system. The diagram shows the protein production pipeline with a description of the antigens that were successfully produced.
*yield as determined by protein band intensity after Coomassie staining.

4.5.2.4 Mass-spectrometry confirmatory analysis

Mass spectrometry analysis was carried out for the eight gamete and ookinete-stage antigens as a second verification of the protein identity. For THX and CHT1, the mass spectrometry was carried out in-house at the KWTRP Mass Spectrometry facility. Peptides generated from each of the purified proteins were sequenced and blasted against a database of the *P. falciparum* proteome as the *P. berghei* database was unavailable. For THX, the peptides positively matched the THX protein ortholog in *P. falciparum*. Unfortunately, no peptides were generated for CHT1, and I was unable to repeat the analysis for verification. For P28, SOAP, PH, VAMP, PBCPP2 and PBCPP3, the analysis was outsourced to the Cambridge Centre for Proteomics for LC/MS/MS analysis. The peptides generated from the six proteins positively matched their respective gene when searched on the *P. berghei* protein database.

4.5.3 Summary of protein production

A summary of the antigens successfully produced and the expression system they were produced in is provided below in **Table 4.5**.

Table 4.5: Summary of antigens successfully produced as recombinant protein

Candidate name	Gene ID	Parasite stage	Parasite species	Expression system*
Pfs230	PF3D7_0209000	Gametocyte	*P. falciparum*	WGCFS
CVMPPP	PF3D7_1314500	Gametocyte	*P. falciparum*	Mammalian
PEB-P	PF3D7_0303900	Gametocyte	*P. falciparum*	Mammalian
PSOP1	PF3D7_0721700	Gametocyte	*P. falciparum*	Mammalian
CPP4	PF3D7_0208800	Gametocyte	*P. falciparum*	WGCFS
MDV1	PF3D7_1216500	Gametocyte	*P. falciparum*	WGCFS
G377B 3D7	PF3D7_1250100	Gametocyte	*P. falciparum*	WGCFS
G377B PfKE04	PF3D7_1250100	Gametocyte	*P. falciparum*	WGCFS
PSOP25 3D7	PF3D7_0620000	Gametocyte	*P. falciparum*	WGCFS
PSOP25 PfKE04	PF3D7_0620001	Gametocyte	*P. falciparum*	WGCFS
P28	PBANKA_0514900	Gamete and ookinete	*P. berghei*	Bacterial
PBCPP2	PBANKA_1463900	Gamete and ookinete	*P. berghei*	Bacterial
PBCPP3	PBANKA_1112700	Gamete and ookinete	*P. berghei*	Bacterial
SOAP	PBANKA_1037800	Gamete and ookinete	*P. berghei*	Bacterial
PH	PBANKA_0417200	Gamete and ookinete	*P. berghei*	Bacterial
CHT1	PBANKA_0800500	Gamete and ookinete	*P. berghei*	Bacterial
THX	PBANKA_0942500	Gamete and ookinete	*P. berghei*	Bacterial
VAMP	PBANKA_1303700	Gamete and ookinete	*P. berghei*	Bacterial
SEAP	N/A	Control	N/A	Mammalian
GST	N/A	Control	N/A	WGCFS

* WGCFS – wheat germ cell-free expression system.

4.6 Discussion

The work described in this chapter outlines the identification of potential target antigens from *Plasmodium* sexual stages for further characterisation by immunoprofiling and transmission-blocking assays. To identify antigens with high expression in the mature stage five gametocyte, I used proteomic rather than transcriptomic data. Owing to the potential for mRNA degradation or delayed mRNA translation (e.g. as known for Pfs25)[361,531,532], proteomic data provides a more accurate picture of stage-specific expression patterns. A total of 25 antigens from the gametocyte stage and 21 antigens from the gamete and ookinete stage were identified. The majority of the identified antigens (19 of the gametocyte antigens and 16 gamete and ookinete antigens) had not been studied as TBV candidates and hence provided attractive targets for study.

As the whole genome sequence of a lab-adapted field isolate from Kilifi was available, I decided to analyse the presence of sequence variation in the target antigens. Antigen polymorphism is a challenge for vaccine design as strain-specific responses limit the efficacy of highly polymorphic vaccine candidates, for instance, the blood-stage antigens AMA1[256] and MSP-2[254]. Though exhibiting less polymorphism than blood stage antigens, Pfs230 and Pfs48/45 contain non-synonymous SNPS and indels[203,425,487,533,534]; hence the impact this variation can have on vaccine efficacy needs to be investigated.

In order to analyse sequence variation in the selected antigens, I compared respective sequences from the *Plasmodium falciparum* strain 3D7 to those from the field strain PfKE04. There was evidence of sequence variation ranging from SNPs to insertions/deletions (indels) for 11 of the gametocyte antigens and only 4 of the gamete and ookinete antigens. As gamete/ookinete antigens are not expressed in the human host, extensive polymorphism resulting from immune pressure (as seen with immunodominant antigens such as AMA1 and MSP-2[367,535,536]) does not occur. Therefore, the level of sequence variation would be lower than that of gametocyte antigens. The most common variation was indels, ranging in size from one amino acid (APP) to 50 amino acids (PSOP25) (**Table 4.1** and **Table 4.3**). SNPs were also present, with four of the gametocyte antigens and one gamete/ookinete antigen having more than one SNP along the sequence.

The AT-rich genome of *P. falciparum* contains extensive low complexity regions characterised by homopolymers of single amino acids, heteropolymers of repeated motifs or irregularly repeated stretches of amino acids[482–484]. Such regions are typically in exposed regions of proteins and are prone to diversification either by deletion, mutation or recombination events, possibly as an immune evasion strategy. The extent to which sequence variation affects the efficacy of a candidate antigen can vary. For instance, the three amino acid deletion in Pfs230-C identified in PfKE04 has also been identified in field isolates in Ghana[425]. The study in Ghana found that 30% of their sequenced samples had this polymorphism. However, the sequence variation did not lead to differential immune responses to Pfs230. I produced both variants of PSOP25 and G377B intending to analyse whether there was an impact on immune recognition (**Chapter 5**) or functional activity of antibodies raised against the proteins (**Chapter 6**).

Initial protein expression trials were carried out in the WGCFS as it has successfully produced *P. falciparum* genes [491,494,537]. Direct cloning of the GOI into the WGCFS expression vector after amplification was unsuccessful and therefore, I chose to use a sub-cloning approach. I first cloned the PCR products into an optimised cloning vector before excising them and cloning them into the expression vector. This approach proved most successful, and I was able to clone 19 of the gametocyte antigens and 15 of the gamete/ookinete antigens. Evidence of protein expression was evident for 14 of the gametocyte antigens, a success rate of 74% which is comparable to that reported by Fan *et al*. (2013)[492] who expressed *P. falciparum* merozoite antigens in the WGCFS. Due to cost and time considerations, I chose to scale up production for 4 of the antigens (CPP4, MDV1, PSOP25 (PfKE04 and 3D7) and G377 (domain B, PfKE04 and 3D7).

As the mammalian system has also successfully produced *P. falciparum* antigens[485,488], I also attempted expression of a subset of proteins in the HEK293E system. Nucleotide sequences corresponding to the GOI were codon optimised and had all potential N-glycosylation sites modified to enhance protein expression[488]. Five of 13 proteins were produced, and only three of these were produced at a high enough yield to allow scale-up. While this was lower than the success rate of 68% reported by Daria *et al*. (2017)[304], I was unable to optimise expression conditions for the unsuccessful antigens owing to time constraints. Therefore, I cannot confidently

comment on their expression potential. Therefore, in total, I was able to produce seven gametocyte antigens (including two antigens with variants) in either system for further evaluation. Additionally, Pfs230-C was included as a positive control bringing the total number of gametocyte antigens to eight.

Curiously, the G377 variants migrated at double their expected molecular weight, and the sequence variation between them was not definitively verified. G377 protein has an abundance of acidic amino acid residues that could result in reduced binding of SDS, and hence they remain insufficiently denatured during electrophoresis[538–540]. Insufficient denaturation may retard their progress during electrophoresis. The expected variation between the G377B variants may not have been observed due to the complete deletion of the 'PLNHEEDNF' motif for 3D7 and acquisition of the PfKE04 SNP. The 3D7 isolate used in this study may have acquired novel mutations over time in continuous culture, distinguishing its sequence from the reference sequence on PlasmoDB. Cultured parasites do acquire mutations over time that allow them to better adapt to *in vitro* culture, for instance, loss-of-function mutations in genes required for sexual stage progression[541]. Nevertheless, the differential migration pattern did indicate a difference between G377B 3D7 and PfKE04, and hence I decided to proceed with the two variants in further assays.

I had a much lower success rate when expressing the gamete and ookinete antigens in the wheat germ system. Few were expressed (5 of 15) and protein yields were lower than with the gametocyte antigens. However, I was able to obtain sufficient yields of CHT1 and THX for further work. Protein expression was reattempted for all antigens in the bacterial expression system. I chose a modified bacterial strain that provides an oxidising environment for disulphide bond formation and is also supplemented with rare tRNAs to overcome codon bias[496,497]. In this way, I hoped to circumvent the challenges of producing eukaryotic proteins in bacterial cells, for instance, insoluble or truncated protein and low success rates, [480,525]. Additionally, I chose to fuse the recombinant protein to a SUMO tag to enhance solubility[529]. Of the 11 antigens tested in small-scale expression trials, eight antigens were expressed, and six of these were expressed at high yield to give a success rate of roughly 73%. This was encouraging and potentially highlights an improved protocol for producing protein in the bacterial expression system where success rates as low as 6.3%[480] or 7%[481] have been reported.

Therefore, the total number of gamete/ookinete antigens for evaluation stood at eight (P28, PH, SOAP, VAMP, PBCPP2, PBCPP3, CHT1 and THX).

4.6.1 Limitations

The target was to produce full-length protein for the gametocyte, gamete and ookinete antigens where possible, for this reason, I set a target to only express proteins smaller than 120 kDa. It was apparent, however, that for the majority of proteins approximately 80 kDa or larger, both cloning and protein expression were unsuccessful in the wheat germ or bacterial systems. Expression success in both systems is inversely correlated with protein size[485,493]; a phenomenon also evident in this work. In retrospect, working with domains of these proteins may have yielded better results. Additionally, I was unable to express proteins with multiple internal transmembrane domains. In these cases, the expression of the longest ectodomains may have been a better option.

Furthermore, scale-up of production was only possible for a subset of the potential antigens expressed in the wheat germ expression system due to the associated costs and time constraints. Several of the potentially more exciting candidates (the majority of the CPPs from the gametocyte antigens) were excluded from further evaluation. However, these can be the subject of future studies. Moreover, due to the relatively large number of proteins targeted for expression and the various expression systems evaluated, and owing to the time constraints of the PhD, cloning and expression conditions for the failed antigens were not extensively optimised. Despite this, a total of 16 antigens for immunological and functional characterisation were produced. Finally, validation of the protein structure was not carried out, and therefore I cannot verify that the recombinant proteins produced assume their native conformation as within the parasite. Time constraints and limited quantities of proteins available made it challenging to complete this work during my PhD, and further work would be required to address this.

4.6.2 Summary of overall findings

In summary, through the exploitation of publicly available *Plasmodium* proteomic datasets and by using a tried-and-tested approach for antigen selection, I identified and prioritised antigens for evaluation as TBV candidates. The majority of the antigens are ‘novel’ and hence provide ideal candidates for pre-clinical evaluation. As

a single, reliable expression platform for heterologous production of *Plasmodium* proteins does not yet exist, I evaluated different systems to maximise expression success. Protein production was challenging, with difficulties arising at gene amplification, construct design, and finally at the protein expression step. Nevertheless, I produced eight gametocyte antigens and eight gamete and ookinete antigens for immunological and functional evaluation.

Chapter 5

Naturally Acquired Immune Responses to the Identified Gametocyte Antigens

5.1. Introduction

Early studies demonstrated that immune responses to a range of gametocyte antigens were readily detectable in the sera of malaria exposed individuals, and developed rapidly after primary infection[193,201,542,543]. The majority of circulating gametocytes are destroyed within the human host before transmission to mosquitoes can occur. This destruction results in a multitude of gametocyte proteins being presented to the host's immune system, subsequently stimulating an immune response[27,332]. The naturally acquired immune response is mostly humoral, and there is evidence that these responses are capable of inhibiting parasite development within the mosquito, thereby interrupting transmission[74,200]. The ability of these immune responses to interrupt the infectious reservoir has stimulated interest in understanding the dynamics of natural immunity to gametocytes as this could inform the development[74] and implementation of transmission-blocking vaccines[222].

Early investigations into NAI to sexual stage antigens, based on studies of Pfs230 and Pfs48/45, observed that not all malaria-exposed individuals made responses to either antigen[201,423,544,545]. The lack of response was not explained by a lack of parasite exposure and seemed to suggest genetic restriction. However, further studies in twins and individuals with similar HLA genotypes showed that the ability to respond to either antigen is not genetically determined[423,546,547]. Potential explanations for the lack of response may relate to genetic variation in Pfs230 and Pfs48/45 leading to isolate-specific responses[423,544], impaired T helper cell function leading to reduced antibody production[423], short-lived antibody responses and/or low immunogenicity of the antigens[547].

Interestingly, Riley *et al*. (1994), in a longitudinal study, demonstrated the existence of stable responses to Pfs230 in adults[423]. However, like some more recent studies[217,218], Graves *et al*. (1988) did not find an association between increasing age and responses to Pfs230 or Pfs48/45[201]. While associations with age have been discrepant, there is suggestion of a maturation of the sexual stage immune response

with repeated parasite exposure [545] as well as increased antibody prevalence[548]. More studies are required to improve our understanding of naturally immunity to *Plasmodium* sexual stages.

Seroepidemiological studies in endemic populations are typically used to understand the acquisition of naturally acquired immunity. Serological status, as defined by the level of antibody responses to key parasite antigens, is used as a marker of individual and population-level exposure to *P. falciparum* infection[469,549–552]. Such studies are useful for exploring temporal changes in transmission intensity or defining transmission hotspots[469,550,553]. A second approach utilises seroepidemiological studies to identify factors associated with carriage of antibodies to *P. falciparum* antigens. Indicators of parasite exposure, such as age, malaria transmission intensity and parasite prevalence, are assessed for associations with immune responses to parasite antigens. The second approach has been widely utilised in studies aimed at exploring naturally acquired anti-gametocyte immunity[200,203,227], and is the approach that I take in this current chapter to explore antibody responses to the antigens identified in **Chapter 4**.

5.2.Rationale

From the seroepidemiological studies on sexual stage antigens carried out so far, based mostly on antibody responses to Pfs230 and Pfs48/45, there exist discrepancies in the associations observed with age, transmission intensity and transmission season[422] (refer to **Chapter 2**, section **2.1**). Therefore, it is crucial to improve our understanding of these associations. Furthermore, I speculate that gametocyte carriage is also likely to impact naturally acquired immune responses to gametocyte antigens. Gametocyte carriage and asexual parasitaemia are rarely explored in seroepidemiological analyses of naturally acquired responses to sexual stage antigens. A host of factors may influence gametocyte carriage (refer to **Chapter 3**, section **3.1**), for example, host genetics, particularly, the haemoglobinopathies conferring protection against severe malaria[128–130]. Based on this premise, I decided to explore an array of factors relating to parasite exposure and gametocyte carriage to dissect the critical factors associated with naturally acquired immune responses to gametocyte antigens.

To contextualize the associations I observe, I also evaluated antibody responses to AMA1, a highly immunogenic asexual stage antigen[203,550,552] widely studied in the context of seroprevalence. Aside from Pfs230, the other gametocyte antigens under investigation have not been profiled; hence trends seen with AMA1 antibody responses could shed some light on associations seen with these antigens. Additionally, I used a preparation of crude extract from a gametocyte culture to serve as a marker of gametocyte exposure. A crude extract prepared from the asexual parasite stages (schizont extract) is a demonstrated marker of exposure to asexual parasites[551,554,555], lending credibility to this approach. The antibody response to gametocyte extract has not been evaluated as a potential marker of recent gametocyte exposure, and hence I sought to investigate its prognostic ability.

Through this analysis, I sought to understand how age, transmission season, parasite carriage as well as factors influencing parasite carriage influence antibody responses to a panel of largely uncharacterised gametocyte antigens. I used as targets the recombinant proteins generated in **Chapter 4** (**Table 4.5**) for this work. First, I hoped to identify potential TBV candidates. The panel of antigens that I investigated included Pfs230, an antigen to which naturally acquired immunity has been well described (**Chapter 2**). I hypothesise that antigens with similar patterns of association may be potential vaccine candidates. Second, I hoped to identify antigens with potential as serological markers of recent exposure to gametocytes. Such antigens could serve as indicators of populations where TBVs could be prioritised[227] or serve as part of control strategies to monitor the effectiveness of TBV implementation. For this work, I used sera from three cohort studies that provided data on different age groups, varied transmission intensities, seasonality and the variation of antibody responses over time.

5.3. Objectives

The main objective of this analysis was to analyse *Plasmodium falciparum* gametocyte-specific surface antigens as targets of naturally acquired antibodies.

5.3.1. Specific Objectives

- Determine the seroprevalence of gametocyte-specific antibodies in individuals naturally exposed to malaria.

- Describe the dynamics of naturally acquired immune responses to *P. falciparum* gametocyte antigens in relation to (1) commonly used markers of parasite exposure (age, transmission intensity, transmission season, and parasite prevalence), and (2) risk factors for gametocyte carriage.
- Explore how antibody responses to the gametocyte antigens relate to infectiousness to mosquitoes.

5.4. Materials and Methods

5.4.1. Materials

A summary of commercially available reagents is provided in **9.4 Appendix 4,** along with recipes for the buffers and solutions used in this work (**9.5 Appendix 5**).

5.4.2. Methods

5.4.2.1. Description of cohorts used for analysis

For these seroepidemiological analyses, samples and data from three cohorts were used. These cohorts included both children and adults, as described below, accounting for a combined sample set of 542 individuals. The KMLRC cohort comprised of three subcohorts of children followed up longitudinally, with cross-sectional surveys carried out to assess asymptomatic *P. falciparum* infections. The AFIRM cohort comprised both children and adults and had sampling carried out in the wet season and the dry season. For the LAMB cohort, a group of adults who tested positive for parasites on screening were recruited. They were sampled over four months at six time points; once every two weeks for the first month, and once a month after that. A map showing the locations of populations sampled is provided in **Chapter 3, Figure 3.1**. A summary of the cohorts is provided in **Table 5.1** below.

Table 5.1: Summary of the cohorts included in the immunoprofiling

Cohort	Location (s)	Study Design	Period of Sample Collection	Population Sampled	Sample Size
KMLRC	Ngerenya and Junju	Cross-sectional surveys	1998 - 2016	Children	272
AFIRM	Junju	Cross-sectional (seasonally spaced)	January 2014 - February 2015	Children and Adults	216

Cohort	Location (s)	Study Design	Period of Sample Collection	Population Sampled	Sample Size
LAMB	Junju	Longitudinal	June 2015 - October 2015	Adults	54

5.4.2.1.(a) Kilifi Malaria Longitudinal Rolling Cohort (KMLRC)

A detailed description of the study design and sampling protocol for the KMLRC cohort has been provided in **Chapter 3,** section **3.4**. A subset of the archived samples from the cohort was selected for immunoprofiling. The samples were selected by first identifying all gametocyte positive individuals over the 1998 – 2016 follow-up period; a total of 364 samples were identified at this point. Two sets of controls matched by age, sex and cohort were then selected, one set was asexual parasite positive but gametocyte negative, and the second set was asexual parasite negative and gametocyte negative. This gave a total sample set of 1092 samples for analysis. Some of the sera samples had been depleted over time, and only a fraction of samples from Ngerenya and Junju locations were available for analysis. Of the 1092 samples, 66 of the gametocyte-positive, 72 of the asexual parasite positive and 134 of the parasite negative samples from the shortlisted samples had sera available for analysis. Therefore, a total of 272 samples from the KMLRC cohort were used for the analysis. As in Chapter 3, Ngerenya was divided into Ngerenya early (1998 – 2001), a period of moderate transmission and Ngerenya late (2002 – 2016), a period of low transmission. In this chapter, Ngerenya and Junju will be referred to as subcohorts of the KMLRC cohort.

5.4.2.1.(b) Measurement of Malaria Transmissibility in Adults and Children (AFIRM)

The AFIRM study was carried out in Junju location, Kilifi, to describe the proportion of mosquito infections in an area of moderate malaria transmission intensity. Details of this cohort have previously been published[72]. The study design was cross-sectional with two surveys carried out, one during the rainy season and one in the dry season. It is important to note that on the Kenyan coast, parasite transmission occurs all year round, although with increased transmission following the onset of the rainy season[158,556]. There is no widely accepted definition of the start and the end of the

malaria transmission season. Therefore, to define the dry and wet seasons, monthly rainfall data collected between 2013 – 2015 was used. Different participants were recruited at each cross-sectional survey and recruitment was carried out between January 2014 and April 2014 for the dry season (additional participants were recruited between January 2015 and February 2015) and between May and December for the rainy season. Children and adults were recruited into the study regardless of parasite status. Samples were collected from the age categories 2 – 5 years, 6 – 18 years and > 18 years in a ratio of 1:1:2 under the hypothesis that older individuals potentially contribute more to the infectious reservoir[72]. The key inclusion criteria were informed consent, age greater than two years, and willingness to provide a single 5 ml venous blood sample. Individuals with acute disease or severe chronic conditions were excluded. At the time of sampling, rapid diagnostic tests were performed using Carestart RDTs®, and individuals who tested positive were given a full course of anti-malarial treatment. Blood drawn from the participants was used to assess infectiousness using membrane feeding assays, following an established protocol[557], and to detect parasites both microscopically and by molecular methods. Infectiousness was defined as the ability of an individual to infect at least one mosquito. Molecular detection and quantification of all parasites was done by *18S* rRNA quantitative nucleic acid sequence-based amplification (QT-NASBA) and *18S* qPCR while specific detection of female gametocytes was carried out by *Pfs25* mRNA QT-NASBA[558,559]. A total of 216 samples (72 from adults, 72 from children over five years and 72 from children under five years) were selected at random from the main AFIRM dataset for analysis.

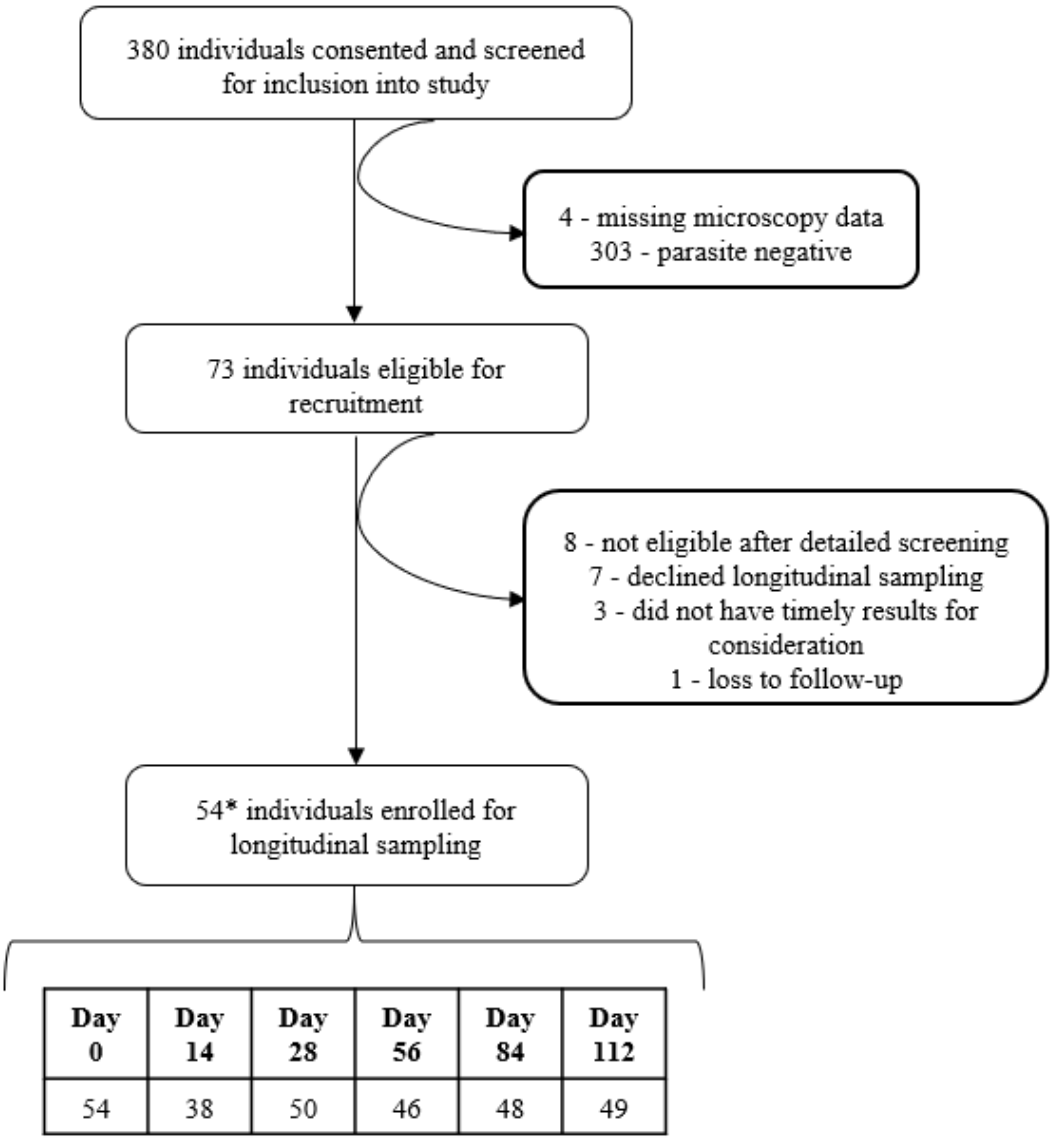
5.4.2.1.(c) Longitudinal Assessment of Malaria Transmissibility (LAMB) Cohort

The LAMB study was a longitudinal analysis of the kinetics of parasite prevalence and infectivity that was carried out in Junju location. Asymptomatic adults were the key population investigated in the LAMB study and were recruited between June to October 2015. The study was designed to collect samples at six time points for analysis: days 0, 14, 28, 56, 84 and 112. At each time point, a venous blood sample was taken (5 ml) for membrane feeding assays using an established protocol[557] and for parasite detection by microscopy and molecular methods. The molecular methods were as follows:*18S* quantitative PCR (*18S* qPCR) for the detection of all parasites,

and *Pfs25* quantitative reverse transcriptase PCR (*Pfs25* qRT-PCR) to detect female gametocytes.

Additionally, the number of parasite clones in each infection, multiplicity of infection (MOI), was determined by genotyping the polymorphic *MSP2* locus by nested PCR. Fluorescently labelled primers targeted to the *IC* and *FC27* regions were used as previously described[560]. This was followed by sizing of the PCR-generated fragments by multi-coloured capillary electrophoresis in a DNA sequencer. The MOI detection was outsourced to the in-house KWTRP sequencing facility.

Inclusion criteria into the study were a minimum of 240 parasites/µl or the presence of gametocytes upon screening. A total of 380 individuals were consented and screened for recruitment into the study. However, after detailed parasitological screening, only 73 individuals were eligible for recruitment. Of the 73 individuals, 19 individuals were excluded from the study for reasons such as withdrawal of consent, loss to follow-up and ineligibility upon further screening. This left 54 individuals for enrolment in the study (**Figure 5.1**). Not all individuals were present for sample collection at each time point due to a variety of challenges. These challenges are listed in the legend to **Figure 5.1**. Therefore, a total of 285 samples were eventually collected at the end of follow-up. A histogram of the number of samples each participant contributed has been added to the supplementary material (**9.9 Appendix 9**).



Day 0	Day 14	Day 28	Day 56	Day 84	Day 112
54	38	50	46	48	49

Figure 5.1 Participant recruitment into the LAMB longitudinal cohort study. Flow diagram showing the selection process of individuals to enrol into the LAMB cohort. Reasons for exclusion are shown at each step.
*Not all participants were able to provide samples at each timepoint for reasons described below:
- Four individuals withdrew consent after enrolment: 2 at Day 0, 1 at Day 14 and 1 at Day 56
- One individual was lost to follow-up
Reasons for non-attendance of scheduled visits included: funeral attendance, family visits outside the study area and refusal by spouse due to household obligations.

5.4.2.2. Preparation of gametocyte extract (GE) and AMA1

A crude extract was prepared from mature gametocytes to analyse gametocyte exposure in the cohorts studied. *P. falciparum* NF54 asexual parasites were cultured in complete culture media in an incubator at conditions of 92% N_2, 3% O_2, 5% CO_2 and temperature of 37ºC. The asexual parasites were maintained at 5% hematocrit and

6% parasitaemia. The culture was synchronised twice by sorbitol treatment on the day preceding gametocyte induction, at 0 – 6 hours post-invasion (determined by the morphology of the infected red blood cell) and at 18 – 24 hours. On the day of induction, day 0, the parasitaemia was diluted to 1% and fresh O^+ red blood cells added to the culture to attain 5% haematocrit. From day one onwards, the media was changed daily with the temperature maintained at 37ºC during media changes. Gametocytaemia was monitored daily, and on day 13, when the gametocytes were between stages IV and V and at 3% gametocytaemia, the gametocyte-infected red blood cells were harvested. As asexual parasites were not cleared after induction, it is possible that low levels of asexual parasites and immature gametocytes were present in the culture at harvest. To prepare the extract, the culture was first spun down at 1800 rpm for 5 minutes to pellet the cells and remove culture media. The pellet was then diluted in carbonate bicarbonate buffer at a ratio of 1:5 and sonicated for 30 minutes. After this, the pellet was rapidly freeze-thawed three times by placing it at -80ºC for 10 minutes, followed by thawing at room temperature for 10 minutes. The prepared extract was then stored at -80 ºC awaiting ELISA.

The AMA1 protein used was expressed in the mammalian HEK293E system and was a kind gift from Dr James Tuju.

5.4.2.3. Enzyme-Linked Immunosorbent Assay (ELISA)

5.4.2.3.(a) Checkerboard titration

To determine the optimum concentration of antigen and dilution of serum to use for the immunoprofiling, checkerboard ELISAs were carried out (**9.8 Appendix 8**). A three-day ELISA was conducted using the ELISA protocol described by Murungi *et al*. (2019)[561] with a few modifications. On the first day, 100 µL of the purified recombinant protein prepared in coating buffer was serially diluted 2-fold from a starting concentration of 4 µg/ml to 0.5 µg/ml. The different dilutions of protein were then coated onto a 96-well Immulon 4 HBX plate such that the lowest dilution (0.5 µg/ml) occupied wells A1-A12, with duplicates on B1-B12, and the highest concentration wells G1-G12 and H1-H12 (**Figure 5.2**). For gametocyte extract, two sets of dilutions were prepared, 1:50 – 1:400 and 1:500 – 1:4000, and these were coated on the plates. The plate, coated with either recombinant protein or gametocyte extract, was then incubated at 4°C overnight. On the next day, the plate was washed

four times with phosphate-buffered saline (PBS) containing 0.05% tween-20 (PBS/T). The plate was then blocked with 200 µL of blocking buffer for five hours at room temperature, and after this, the plates were washed three times with PBS/T.

A previously defined and characterised pool of hyper-immune sera (PHIS) obtained from a random selection of adult residents of Junju location, Kilifi[182,187], and a pool of non-exposed sera from European adults (non-immune sera, PNIS) was used as positive and negative controls respectively. The adults from Junju location are presumed to have had high exposure to malaria and hence possess NAI to malaria. While transmission-blocking activity has not been tested for PHIS, it is strongly reactive to asexual stage antigens and has shown evidence of recognition of the gametocyte antigens MDV1, CPP3 and CPP5 (**9.9 Appendix 9**). The two control sera were prepared in blocking buffer at dilutions of 1:100, 1:200, 1:400, 1:800 and 1:1000 before 100 µl of each dilution of PHIS was added sequentially (from lowest to highest dilution) from wells A1-H1 up to A5-H5. 100 ul of blocking buffer was added to wells A6-H6 and A12-H12 to serve as an assay control (blank). The PNIS, diluted similarly to the PHIS, was added sequentially from wells A7-H7 to A11-H11 and the plate then incubated at 4°C overnight.

On the third day, the plate was washed three times in PBS/T before 100 µL of secondary antibody (polyclonal rabbit anti-human IgG-HRP) was added at a dilution of 1: 5,000 and the plate incubated at room temperature for three hours. The plate was then washed four times, and 100 µL of o-phenylenediamine dihydrochloride (OPD) substrate was added, and the plate incubated at room temperature for 15 minutes. Colour development was stopped by adding 25 µL of 2 M sulphuric acid (H_2SO_4). Absorbance was then read at 492 nM to determine the optical density (O. D) of each sample.

A

		PHIS						NIS					
		1 in 100	1 in 200	1 in 400	1 in 800	1 in 1000	Blank	1 in 100	1 in 200	1 in 400	1 in 800	1 in 1000	Blank
Antigen		1	2	3	4	5	6	7	8	9	10	11	12
0.5ug/ml	A												
	B												
1ug/ml	C												
	D												
2ug/ml	E												
	F												
4ug/ml	G												
	H												

B

		PHIS						NIS					
		1 in 100	1 in 200	1 in 400	1 in 800	1 in 1000	Blank	1 in 100	1 in 200	1 in 400	1 in 800	1 in 1000	Blank
GE		1	2	3	4	5	6	7	8	9	10	11	12
1 in 50	A												
	B												
1 in 100	C												
	D												
1 in 200	E												
	F												
1 in 400	G												
	H												

Figure 5.2: Template used for the checkerboard ELISA. A) Template used for checkerboard titration of the different recombinant antigens. B) Template used for checkerboard titration of gametocyte extract. Two sets of dilutions were prepared, 1 in 50 to 1 in 400, and 1 in 500 to 1 in 4000. The dilutions 1 in 500 to 1 in 4000 were plated in a similar format to the 1 in 50 to 1 in 400 dilutions. GE – gametocyte extract; PHIS – pooled hyperimmune sera; PNIS – pooled non-immune sera.

5.4.2.3.(b) Standardised ELISA

To determine the reactivity of the serum samples to the antigens, gametocyte extract, and AMA1, a standardised ELISA was run using a similar protocol to the checkerboard ELISA. In this case, however, a single concentration of antigen (1 µg/ml for the gametocyte antigens, 0.5 µg/ml for AMA1 and 1 in 250 dilution for GE) was coated onto the 96-well ELISA plate (**Figure 5.3**). A standardised ELISA was chosen to facilitate comparisons of data from different ELISA plates[562]. Additionally, a single dilution of test sera was tested, being: 1 in 200 (or 1 in 100 for lowly reactive antigens) for the gametocyte antigens; 1 in 1000 (or 1 in 2000 for adult sera tested) for AMA1, and 1 in 500 for GE). Four individual non-immune sera (NIS) were tested alongside the test sera as negative controls at the start of the ELISAs. However, these were increased to eight when more non-immune serum samples became available. The NIS was tested against measles antigen to verify that they were indeed reactive (**9.8 Appendix 8**). Two positive controls were used, PHIS and PGP (a pool of 80 sera from

gametocyte-positive individuals from the AFIRM cohort, including 36 of the test samples, with high gametocytaemia as determined by QT- NASBA). A serial dilution of malaria immune globulin (MIG) was also included on each plate to allow for relative quantification of antibody levels in arbitrary units. The MIG used was prepared from the purified immunoglobulin G (98% IgG) of a pool of 834 Malawian adult sera originally manufactured for use as therapy in children with cerebral malaria[563]. All samples were run in duplicate, with duplicates run on a separate plate.

	1	2	3	4	5	6	7	8	9	10	11	12
A	2880	960	320	106.67	35.56	11.85	3.95	1.32	0.44	0.15	0.05	BLK
B	UK 1	Sam106	Sam189	Sam025	Sam003	UK 5	Sam049	Sam142	Sam080	Sam031	Sam002	Sam161
C	Sam029	Sam067	Sam002	Sam056	Sam063	Sam027	Sam017	Sam154	Sam009	PHIS	Sam110	Sam101
D	Sam111	UK 2	Sam113	Sam196	Sam035	Sam085	UK 6	Sam014	Sam064	Sam055	Sam016	Sam143
E	Sam038	Sam099	Sam075	Sam068	Sam078	Sam049	Sam081	Sam107	Sam171	Sam020	PGP	Sam103
F	Sam093	Sam097	UK 3	Sam005	Sam064	Sam042	Sam058	UK 7	Sam076	Sam029	Sam197	Sam044
G	Sam030	Sam135	Sam050	Sam103	Sam060	Sam014	Sam118	Sam055	Sam167	Sam190	Sam049	Sam158
H	Sam086	Sam013	Sam100	UK 4	Sam018	Sam093	Sam180	Sam090	UK 8	Sam046	Sam036	Sam091

Figure 5.3: Template used for the standard ELISA. Sam – sample; UK – sera from European malaria naïve individuals; PHIS – pooled hyperimmune sera; PGP – pooled gametocyte-positive sera; highlighted in blue is the serial dilution of malaria immune globulin (MIG) used to prepare a standard curve for relative quantification.

5.4.2.4. Statistical Analysis

All samples were run in duplicate, and therefore before analysis, the mean OD and co-efficient of variation were calculated. Samples with a coefficient of variation higher than 20% were repeated. Where samples were repeated, only the measurements of the repeat duplicate measurements were used in the analysis. A four-parameter hyperbolic standard curve was generated from the serially diluted MIG present on each plate. The antibody concentration of each sample was then extrapolated from the MIG curve run on the same plate. In order to determine seropositivity to each antigen, a cut-off was calculated by taking the mean antibody concentration of the NIS plus three standard deviations for increased stringency[422] (refer to **Chapter 2**, section **2.6**). A two proportions z-test was used to compare seroprevalence estimates between G377B variants and also between sampling seasons. Additionally, the Cochrane-Armitage test was used to test for a linear trend in proportions. For comparisons of the demographic characteristics of participants from the different cohorts a two proportions z-test was used for the AFIRM cohort, a three proportions test for the

KMLRC subcohorts and a 6 proportions test for the LAMB cohort. For summaries of parasite density in relation to the variables age, sickle cell status, a-thalassaemia status, cohort, season and sampling timepoint, comparisons between groups were tested for significance using the Kruskal-Wallis test where variable had more than one category. If the test was significant, post-hoc analysis using the Dunn's test with Bonferroni correction was used for pairwise comparisons. Where a categorical variable had only two groups, the Wilcoxon rank-sum test was used.

Linear regression models were used to assess the factors associated with the levels of antibodies to the gametocyte antigens. The models were used to assess how well the covariates age, parasite prevalence, sickle cell genotype, α-thalassaemia genotype, transmission intensity, and season predicted the magnitude of antibody responses to the antigens, as well as the breadth of response. Age was used as a categorical variable with the '0 – 5-year' age group chosen as the reference category. As the KMLRC cohort included repeated measures for individuals, I calculated cluster-robust standard errors with clustering specified to occur by participant ID. Owing to the small sample size of the LAMB cohort, a linear mixed effects model, with participant ID included as a random effect was used. Both univariable and multivariable models were analysed, and these included all the variables under investigation. No backwards selection was carried out to assess the associations seen with all the variables and examine how these associations varied across antigens.

To assess associations the factors associated with infectivity to mosquitoes, the frequency of infectiousness (with infectiousness coded as a binary variable) was tabulated according to the variables age and parasite status. A Fisher's exact test used to test for significant differences among the categories of the variables.

5.5. Results

5.5.1. Demographic characteristics of study participants

5.5.1.1. KMLRC

The demographic characteristics of the study participants are presented in **Table 5.2.** The majority of observations were from children aged 0 – 5 years of age in all subcohorts (Ngerenya early - 64%, Ngerenya late - 60% and Junju - 52%, $p = 0.3017$). Asexual parasite prevalence was 78% in Ngerenya early, 60.4% in Junju and 19% in Ngerenya late. Gametocyte prevalence was 25% in Ngerenya early, 33% in Junju and 8% in Ngerenya early. Though cases were initially matched with controls (based on age, sex and cohort), the matching was lost after sample retrieval. Thus, this is reflected in the variable prevalence of asexual parasites in the sub-cohorts, with a disproportionate number of controls in Ngerenya late. The prevalence of sickle cell trait genotype (AS) was quite varied among the subcohorts with the lowest prevalence in Ngerenya early 2%, with higher prevalence in Ngerenya late 17.6% and Junju 27% ($p = 0.0009$). The higher prevalence of sickle cell trait in Ngerenya late likely results from a loss in matching after sample retrieval where a majority of Ngerenya early samples were missing and not an actual increase in prevalence over time. On the other hand, close to half the participants were heterozygous for α – thalassaemia (Ngerenya early 47.7%, Ngerenya late 52.8% and Junju 50%, $p = 0.43$).

Table 5.2: Demographic characteristics of observations from a subset of the KMLRC cohort study participants

	Subcohort			
	Ngerenya		Junju	*p* – value*
	Early	Late		
Total number of observations	50	126	96	
Number of observations from females (%)	25 (50.0)	40 (31.7)	45 (46.9)	**0.0235**
Number of observations per age group (%)				
0 - 5 years	32 (64.0)	76 (60.3)	50 (52.1)	0.3017
6 - 10 years	14 (28.0)	41 (32.5)	29 (30.2)	0.8281
11-15 years	4 (8.0)	9 (7.1)	17 (17.7)	**0.0339**
Temperature (°C), median (IQR)	.	36.9 (36.6 - 37.1)	36.5 (36.2 - 36.9)	
Number of asexual parasite positive observations (%)	39 (78.0)	24 (19.0)	58 (60.4)	**<0.0001**
Number of gametocyte positive observations (%)	25 (50.0)	8 (6.3)	33 (34.4)	**<0.0001**
Number of observations with sickle genotype (%)				
AA	49 (98.0)	103 (82.4)	70 (72.9)	**0.0009**
AS	1 (2.0)	22 (17.6)	26 (27.1)	**0.0009**
Number of observations with α-thalassaemia genotype (%)				
Normal	18 (40.9)	39 (31.7)	31 (32.3)	0.8566
Heterozygous	21 (47.7)	65 (52.8)	48 (50.0)	0.4329
Homozygous	5 (11.4)	19 (15.4)	17 (17.7)	0.4678
Missing data				
Sickle genotype	.	1	.	
α-thalassaemia genotype	6	3	.	
Temperature	50	.	.	

* A three proportions Z-test was used to compare the proportions among the KMLRC subcohorts

5.5.1.2. AFIRM

There were slightly higher numbers of participants sampled in the wet season, 120 participants compared to 96 participants sampled in the dry season (**Table 5.3)**. However, there was no statistically significant difference in the representation of participants from each of the age categories for both the dry and wet seasons. Parasite prevalence was higher in the wet season compared to the dry season when detection was carried out using rapid diagnostic tests, 28.3% vs 12.5% in the wet and dry seasons respectively (p = 0.0078). No difference was observed between parasite carriage in the dry and the wet season when molecular methods were used (*18S* qPCR: 32.5% vs 41.7% (p = 0.21) and NASBA *18S*: 49.2% vs 50% (p = 1)), indicating a high proportion of submicroscopic parasite carriage in the dry season. *18S*A similar pattern was indicated for gametocyte prevalence, as detected by female-gametocyte specific NASBA *Pfs25* (24% vs 25.8% in the wet and dry seasons respectively (p = 0.87)). Additionally, for parasite detection by microscopy, no significant difference in parasite carriage was observed in the dry and the wet season (16.7% vs 8.3% (p = 0.11) for asexual parasites and 1% vs 3.3 % (p = 0.51) for gametocytes in the wet and dry seasons respectively). The prevalence of sickle cell trait was lower than that of α-thalassaemia (17.4% on average), with approximately half the participants heterozygous for α – thalassaemia. There was no statistically significant difference in the proportion of participants with the different sickle cell and a-thalassaemia genotypes in the dry compared to the wet season.

Table 5.3: Demographic characteristics of participants from the AFIRM cohort study

	Season		
	Dry	**Wet**	***p* – value***
Total number	96	120	
Number of females (%)	61 (63.5)	61 (50.8)	0.0829
Number per age group (%)			
0 - 5 years	27 (28.1)	45 (37.5)	0.1912
6 - 15 years	33 (34.4)	39 (32.5)	0.8845
>15 years	36 (37.5)	36 (30)	0.3093
Temperature (°C), median (IQR)	36.6 (36.3 - 37.0)	36.6 (36.2 - 36.8)	
Number RDT positive (%)	12 (12.5)	34 (28.3)	**0.0079**
Parasite Prevalence			
qPCR (18s)	40 (41.7)	39 (32.5)	0.2121
NASBA (18s)	48 (50)	59 (49.2)	1.0000
Asexual parasite prevalence - Microscopy (%)	8 (8.3)	20 (16.7)	0.1078
Gametocyte prevalence (%)			
Microscopy	1 (1.0)	4 (3.3)	0.5108
NASBA (Pfs25)	23 (24)	31 (25.8)	0.8744
Sickle genotype (%)			
AA	77 (80.2)	102 (85)	0.4550
AS	19 (19.8)	18 (15)	0.4550
α - Thalassaemia genotype (%)			
Normal	30 (31.3)	38 (31.7)	1.0000
Heterozygous	51 (53.1)	56 (46.7)	0.4200
Homozygous	15 (15.6)	26 (21.7)	0.3418

* A two-proportions Z-test was used to compare the proportions between the dry and wet seasons.

5.5.1.3. LAMB

A total of 54 adults, mostly female (88%, median age 29.5 years), were recruited on screening, though not all were present for sampling at each time point (**Table 5.4)**. All individuals considered for the study were positive for asexual parasites upon screening. However, on day 0, the day of enrolment, only 16% had microscopically detectable parasites with 48.1% positive by *18S* qPCR. No treatment was provided to the participants prior to the study. Over the follow-up period, parasitaemia as detected by RDT varied significantly over the timepoints (p = 0.03). However, no significant variation in parasite prevalence over the timepoints was observed when parasite detection was by qPCR (p = 0.95). Asexual parasite prevalence as detected by microscopy also did not vary significantly over the 6 timepoints of follow up (p = 0.21). For gametocytaemia, only one individual tested positive for gametocytes by microscopy during the follow-up period, and this was at day 14. Molecular detection by qRT-PCR detected more gametocyte carriers and indicated significant variation in gametocyte prevalence over the timepoints (p = 0.01). The frequencies of the different sickle cell and α-thalassaemia genotypes were similar to those described for the AFIRM cohort and were not significantly different between timepoints (p >0.05)

Table 5.4: Demographic characteristics of participants from the LAMB cohort study

	Day of Follow-up							
	Screening	**Day 0**	**Day 14**	**Day 28**	**Day 56**	**Day 84**	**Day 112**	***p* – value***
Total number	54	54	38	50	46	48	49	
Age (years), median (IQR)	29.5 (23 - 43)	29.5 (23 - 43)	30.5 (23 - 40)	31 (26 - 44)	31 (25 - 43)	31 (25 - 43)	31 (25 - 44)	
Number of females (%)	47 (88)	47 (87)	34 (89.5)	43 (86.0)	41 (89.1)	41 (85.4)	42 (85.7)	0.9874
Temperature (°C), median (IQR)	36.7 (36.4 - 36.8)	36.4 (36 - 36.7)	36.2 (35.9 - 36.8)	36.3 (36 - 36.5)	36.3 (35.9 - 36.6)	36.4 (36 - 36.7)	36.4 (36 - 36.6)	
Number RDT positive (%)	ND	22 (40.7)	17 (44.7)	11 (22.0)	9 (19.6)	13 (27.1)	11 (22.4)	**0.0293**
Parasite prevalence – *18S* qPCR (%)	ND	26 (48.1)	21 (55.3)	23 (46.0)	21 (48.8)	25 (52.1)	24 (49.0)	0.9468
Asexual parasite prevalence - Microscopy (%)	54 (100)	9 (16.7)	9 (25.7)	4 (8)	9 (20.9)	5 (10.6)	10 (20.4)	0.2139
Gametocyte prevalence (%)								
Microscopy	3 (5.6)	0	1 (2.9)	0	0	0	0	0.2230
qPCR	ND	11 (20.4)	8 (21.1)	8 (16)	1 (2.2)	2 (4.2)	4 (8.2)	**0.0098**
Sickle genotype (%)								
AA	42 (77.8)	42 (77.8)	28 (73.7)	39 (78.0)	35 (76.1)	38 (79.2)	39 (79.6)	0.9889
AS	12 (22.2)	12 (22.2)	10 (26.3)	11 (22.0)	11 (23.9)	10 (20.8)	10 (20.4)	0.9889
α - Thalassaemia genotype (%)								
Normal	22 (41.5)	22 (41.5)	14.0 (37.8)	21 (42.9)	19 (42.2)	20 (42.6)	20 (41.7)	0.9981
Heterozygous	24 (45.3)	24 (45.3)	18 (48.6)	22 (44.9)	21 (46.7)	21 (44.7)	22 (45.8)	0.9993
Homozygous	7 (13.2)	7 (13.2)	5 (13.5)	6 (12.2)	5 (11.1)	6 (12.8)	6 (12.5)	0.9996
Number with anaemia (%)	ND	19 (35.2)	11 (28.9)	17 (34.0)	18 (39.1)	15 (32.6)	21 (42.9)	0.7996
Missing Data								
Microscopy	.	.	3	.	3	1	.	
α - Thalassaemia genotype	1	1	1	1	1	1	1	
Anaemia	.	.	.	.	.	2	.	

	Day of Follow-up							
	Screening	**Day 0**	**Day 14**	**Day 28**	**Day 56**	**Day 84**	**Day 112**	***p* – value***
Age	4	4	2	3	3	3	3	
Total number	54	54	38	50	46	48	49	

* A six-proportions Z-test was used to compare proportions between the sampling timepoints.

5.5.2. Seroprevalence to gametocyte antigens

Immune responses were measured to the gametocyte antigens that were identified and produced successfully as recombinant protein (**Chapter 4,** section **4.6**). Additionally, antibody responses were measured to AMA1 (a known marker of exposure to asexual parasites[203,550,552]) and GE (a potential marker of exposure to gametocytes). The analysis was limited to the AFIRM cohort, where sampling was carried out for all age groups (children and adults). The median seroprevalence to the gametocyte antigens was 59.5% ranging from 38.9% for PSOP1 to 76.9% for G377B 3D7 (**Table 5.5**). Seroprevalence to G377B 3D7 and to G377B PfKE04 (71.3%) was relatively high, with no difference in seroprevalence to the two variants (p = 0.23, 2-proportions Z-test). While antibody responses to Pfs230 were not the most prevalent 42.6% of the population studied had responses to Pfs230. Antibody responses to GE were highly prevalent with seroprevalence estimates of 87.8% closely matching estimates to AMA1 (87.9%).

Table 5.5: Seroprevalence of immune responses to the gametocyte antigens, AMA1 and gametocyte extract

Antigen	N[a]	Seropositive	Prevalence (%)
Pfs230	148	63	42.6
CVMPPP	216	120	55.6
PEB-P	216	125	57.9
PSOP1	216	84	38.9
CPP4	216	127	58.8
MDV1	216	130	60.2
G377B 3D7	216	166	76.9
G377B PfKE04	216	154	71.3
AMA1	215	189	87.9
Gametocyte Extract	205	180	87.8

[a] Antibody responses to PfKE04 were not measured for the AFIRM cohort owing to depletion of protein.
[b] Total number of samples assayed for each antigen; the total AFIRM sample set consisted of 216 individuals. However, not all samples were tested for each antigen as antigen quantities were limited and did not allow testing the entire sample set for each antigen.

In an age-stratified analysis, with age divided into three categories: 0 – 5 years, 6 – 15 years and >15 years, there was a significant trend towards increasing seroprevalence with age for all antigens except for CPP4 (p = 0.06, **Figure 5.4)**. As for season there

was no difference in seroprevalence between the dry and the wet seasons for any of the antigens (**Figure 5.5).**

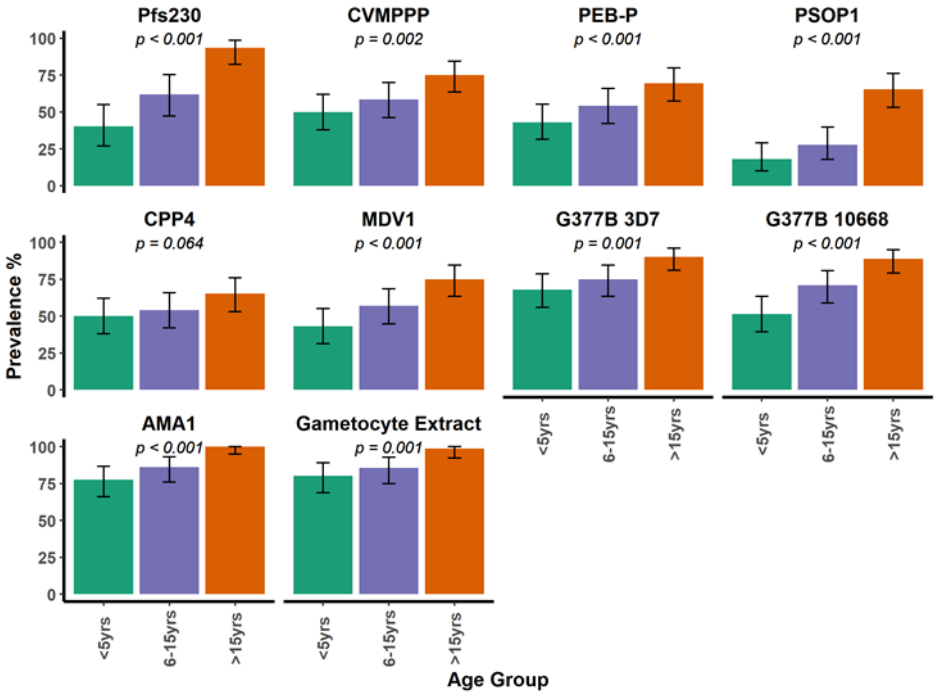


Figure 5.4: Seroprevalence to the gametocyte antigens, AMA1 and gametocyte extract in the AFIRM cohort analysed by age group. Bar plots showing the prevalence of antibodies to the various gametocyte antigens, AMA1 and gametocyte extract within the different age categories. The Cochran-Armitage test for trend was used to test for a linear trend in increasing seroprevalence with age, respective *p*-values are presented at the top of each panel. Error bars show 95% binomial confidence intervals (Clopper–Pearson interval).

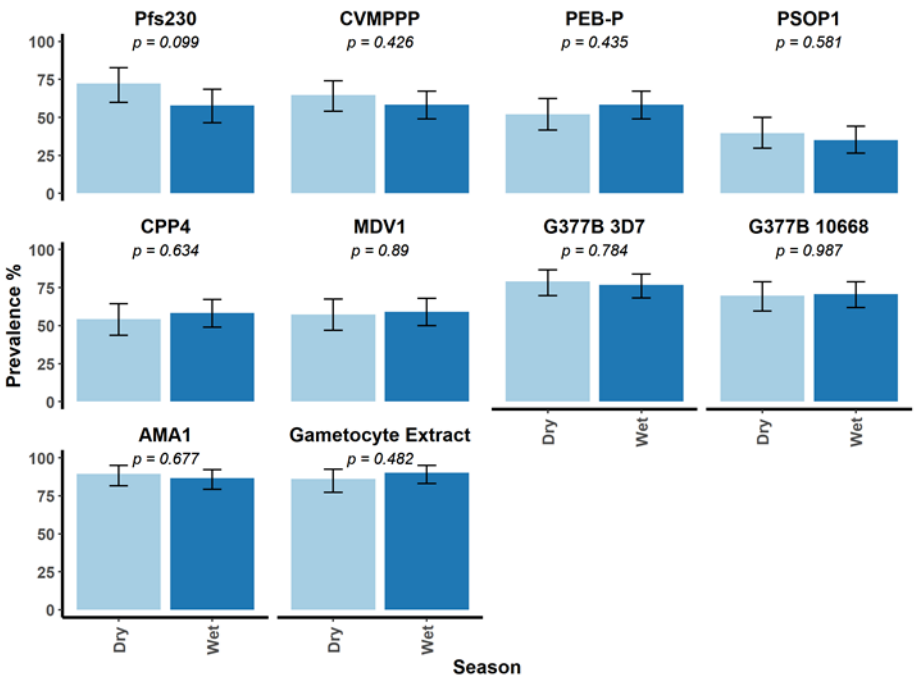


Figure 5.5: Seroprevalence to the gametocyte antigens, AMA1 and gametocyte extract in the AFIRM cohort analysed by season. Bar plots showing the prevalence of antibodies to the various gametocyte antigens, AMA1 and gametocyte extract in the dry and the wet seasons. A two-proportions z-test was used to compare proportions in the dry and wet seasons. Respective *p* values are presented at the top of each panel. Error bars show 95% binomial confidence intervals (Clopper–Pearson interval).

5.5.3. Factors associated with the magnitude of antibody response to the gametocyte antigens

Next, I assessed how determinants of parasite exposure (age, parasite prevalence, season and transmission intensity) as well as host genetic factors (sickle and α – thalassaemia genotypes) relate to the levels of antibodies against the gametocyte antigens. I selected these variables based on literature reporting associations with gametocyte carriage or gametocyte-specific immune responses[68,70,79,227,415]. Furthermore, age, parasite prevalence and transmission intensity were also identified as prognostic indicators of gametocytaemia from the epidemiological analysis I carried out in **Chapter 3**.

5.5.3.1. Relationship between parasitaemia and possible factors influencing anti-gametocyte immune responses

Before analysing predictors of immune responses to the gametocyte antigens, I investigated how some of the covariates above (age, sickle cell genotype, α-thalassaemia genotype, transmission intensity (subcohort) and season) relate to asexual parasite and gametocyte densities. For this, I analysed data from the KMLRC and AFIRM cohorts. Parasite densities (asexual parasite and gametocyte) were determined microscopically for the KMLRC and did not differ between the sickle or α-thalassaemia genotypes (**Figure 5.6 A and B)**. When looking at the relationship between age and parasite densities, higher asexual parasite densities were recorded in children aged 0 – 5 years compared to their counterparts aged 6 – 15 years ($p = 0.02$) (**Figure 5.6 C**)). However, gametocyte densities were similar across all age groups. Additionally, parasite densities (asexual parasite or gametocyte) did not differ among the sub-cohorts (**Figure 5.6 D**).

Few participants from the AFIRM cohort had microscopically detectable gametocytes, limiting a robust analysis. However, an analysis of relationships between the aforementioned covariates and sub-microscopic parasite densities was possible. From the analysis, there were no differences in sub-microscopic parasite densities across the sickle or α-thalassaemia genotypes, as also seen with the KMLRC cohort (**Figure 5.7** A and B). Additionally, sub-microscopic parasite densities did not vary with age groups (**Figure 5.7** C). For season, there were higher parasite densities in the wet season, and this was statistically significant for both all parasites ($p = 0.02$) and gametocytes ($p = 0.01$) (**Figure 5.7** D).

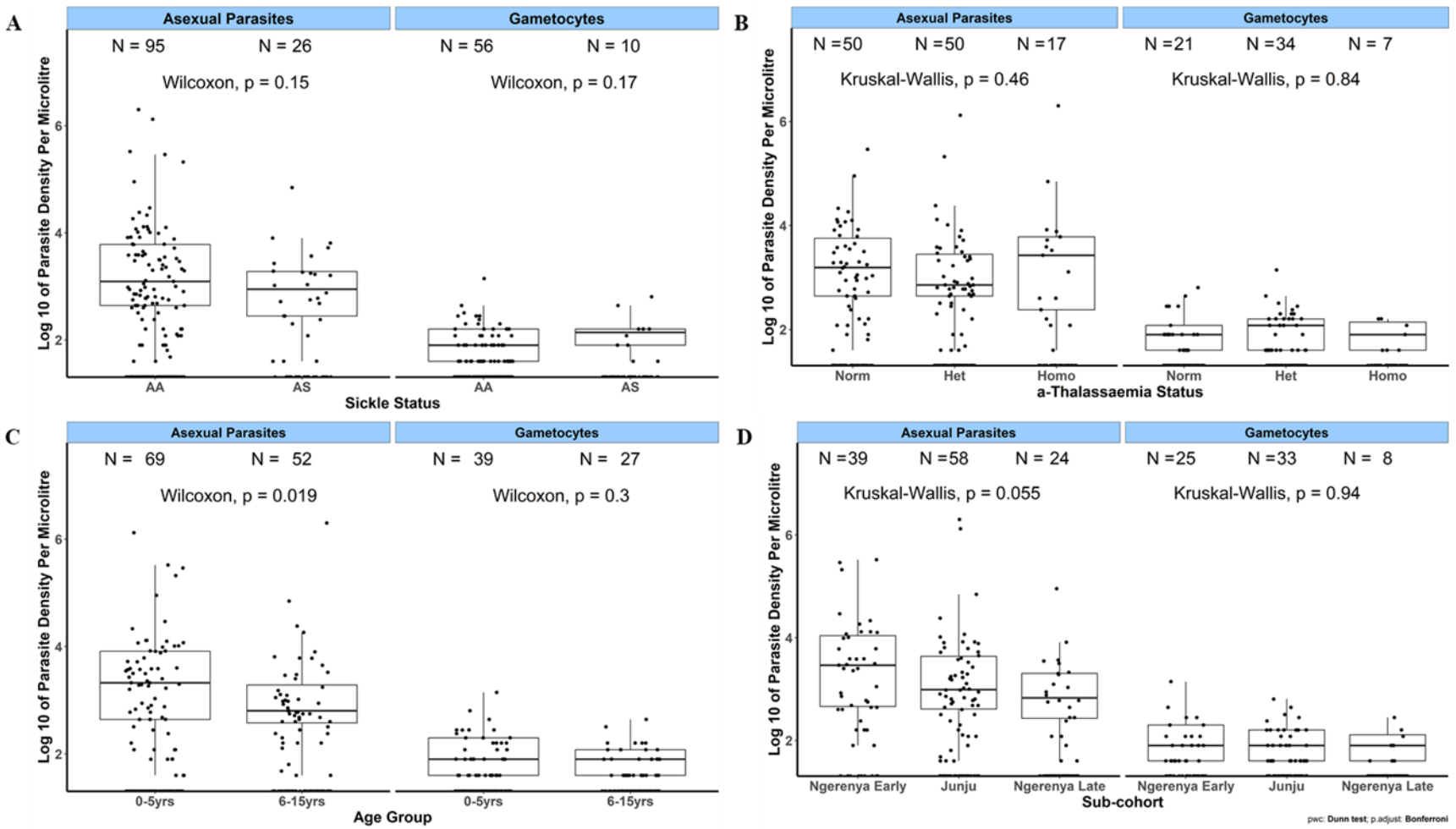


Figure 5.6: Variation in parasite density categorised by factors predictive of gametocyte carriage in the KMLRC cohort. Asexual parasite and gametocyte densities by A) Sickle genotype, B) α-thalassaemia genotype, C) Age group, D) Sub-cohort (moderate transmission (Ngerenya early and Junju) and low transmission (Ngerenya late)) with

parasitaemia detected by microscopy. Comparisons carried out by Wilcoxon test and Kruskal-Wallis test (post-hoc analysis after Kruskal-Wallis carried out using Dunn's test with Bonferroni correction). Number of parasite positive individuals (N) is included at the top of each graph for each category. α-thalassaemia: Norm – normal, Het – heterozygous, and Homo – homozygous. The boxes of boxplots display the median bound by the first and third quartiles, with the whiskers depicting the lowest and highest values (excluding outliers). The dots indicate individual datapoints.

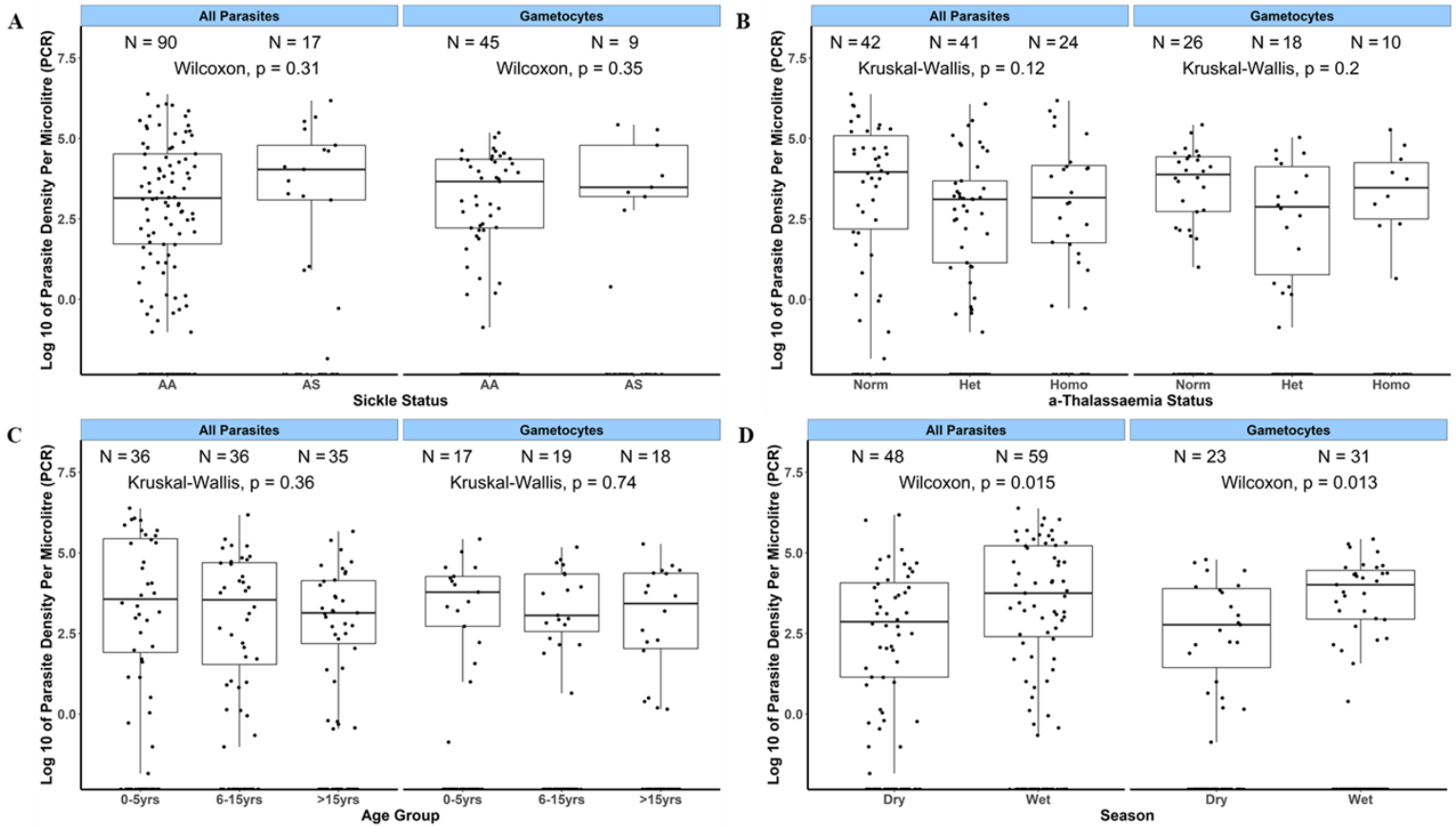


Figure 5.7: Variation in parasite density categorised by factors predictive of gametocyte carriage in the AFIRM cohort. Asexual parasite and gametocyte densities by A) Sickle genotype, B) α-thalassaemia genotype, C) Age group, D) Season with parasitaemia detected by *18S* NASBA (all parasites) and *Pfs25* NASBA (female gametocytes). Comparisons carried out by Wilcoxon test and Kruskal-Wallis test. Number of parasite positive individuals (N) is included at the top of each graph for each category. α-thalassaemia: Norm – normal, Het – heterozygous, and Homo – homozygous. The boxes of boxplots display the median bound by the first and third quartiles, with the whiskers depicting the lowest and highest values (excluding outliers). The dots indicate individual datapoints.

5.5.3.2. Factors associated with the magnitude of antibody response to the gametocyte antigens: KMLRC cohort

I explored the relationship between age, parasitaemia (asexual and gametocyte positivity (as detected by microscopy)), sickle and α-thalassaemia genotypes, and the magnitude of antibody responses to the gametocyte antigens. Additionally, I evaluated how transmission intensity relates to anti-gametocyte antibody responses. Linear regression models were used to predict associations with antibody concentration from the specified covariates, with age included as a categorical variable. Associations seen in the univariable analysis are in **9.9 Appendix 9,** while the multivariable analyses are discussed below. As matching of cases and controls was lost owing to the depletion of some of the initially selected samples for the KMLRC cohort analysis (**5.4.2.1.(a)**), the results of the univariable analysis would be confounded and hence only the results of the multivariable analysis are interpreted.

In the multivariable analysis (**Table 5.6**), a significant association between concurrent asexual parasitaemia and increased magnitude of antibody response was observed for all antigens. Conversely, gametocyte positivity was significantly associated with increased antibody responses to AMA1, GE, CVMPPP, PEB-P, and CPP4 (p <0.05). Additionally, statistically significant associations between increasing age and increased immune responses to AMA1, Pfs230, G377B 3D7 and G377B PfKE04 were observed. For sickle cell trait, no association with antibody concentration was observed for any of the antigens. On the other hand, α-thalassaemia heterozygosity (estimate -0.24, 95% CI: -0.45, -0.03, p = 0.02) and homozygosity (estimate -0.3, 95% CI: -0.57, -0.04, p = 0.03) were associated with reduced responses to GE. From the multivariable analysis, a clear association with transmission intensity was not evident. Residing in Ngerenya late was only associated with reduced immune responses to GE (estimate -0.30, 95% CI: -0.54, -0.06, p = 0.02) and G377B 3D7 (estimate -0.12, 95% CI: -0.23, -0.004, p = 0.04).

For both G377B variants, similar associations between increasing age and increased antibody responses as well as increased antibody responses with concurrent parasitaemia were observed. Additionally, predictive models for both variants had similar R^2 values (G377B 3D7 R^2 = 0.36, G377B 10668 R^2 = 0.36). Models predicting the magnitude of antibody responses to the PSOP25 variants did not show associations

with age, with only PSOP25 PfKE04 showing an association with concurrent asexual parasitaemia. The R^2 value for the model predicting the magnitude of antibody responses to PSOP25 PfKE04 ($R^2 = 0.18$) was marginally higher than that of PSOP25 3D7 ($R^2 = 0.16$).

Table 5.6 Multivariable linear regression analysis of the factors predicting the magnitude of antibody response to the gametocyte antigens, AMA1 and gametocyte extract – KMLRC cohort

Covariate	Pfs230			CVMPPP			PEB-P			PSOP1		
	Estimate	**95% CI**	***p* value**	**Estimate**	**95% CI**	***p* value**	**Estimate**	**95% CI**	***p* value**	**Estimate**	**95% CI**	***p* value**
Age Group												
0 - 5 years	0.00	.	.	0.00	.	.	0.00	.	.	0.00	.	.
6 - 10 years	0.15	0.04, 0.25	**0.0056**	0.06	-0.05, 0.17	0.2857	0.02	-0.09, 0.14	0.6728	0.05	-0.07, 0.17	0.4236
11 - 15 years	0.17	0.03, 0.31	**0.0193**	0.06	-0.13, 0.25	0.5172	-0.02	-0.18, 0.14	0.8106	0.13	-0.08, 0.34	0.2312
Asexual parasite positive	0.25	0.12, 0.39	**0.0003**	0.28	0.14, 0.42	**0.0001**	0.28	0.14, 0.42	**0.0001**	0.17	0.01, 0.33	**0.0418**
Gametocyte positive	0.02	-0.11, 0.15	0.7498	0.18	0.03, 0.34	**0.0225**	0.17	0.01, 0.33	**0.0353**	0.08	-0.09, 0.24	0.3520
Sickle			.									
Normal	0.00	.	.	0.00	.	.	0.00	.	.	0.00	.	.
Heterozygous	0.00	-0.12, 0.12	0.9801	-0.01	-0.14, 0.11	0.8159	0.05	-0.07, 0.18	0.4088	0.06	-0.08, 0.21	0.3977
α-thalassaemia			.									
Normal	0.00	.	.	0.00	.	.	0.00	.	.	0.00	.	.
Heterozygous	-0.01	-0.12, 0.09	0.8245	0.06	-0.06, 0.18	0.2926	-0.04	-0.15, 0.07	0.5284	-0.01	-0.13, 0.11	0.9114
Homozygous	-0.03	-0.17, 0.11	0.6879	-0.05	-0.2, 0.09	0.4653	-0.01	-0.16, 0.14	0.9069	0.13	-0.07, 0.33	0.2082
Subcohort			.									
Junju	0.00	.	.	0.00	.	.	0.00	.	.	0.00	.	.
Ngerenya early	-0.15	-0.3, 0.01	0.0679	-0.05	-0.24, 0.14	0.5965	-0.03	-0.21, 0.14	0.7291	0.04	-0.16, 0.24	0.6904
Ngerenya late	-0.03	-0.15, 0.09	0.6353	0	-0.13, 0.13	0.9848	0	-0.12, 0.13	0.9530	-0.03	-0.19, 0.13	0.7003
Covariate	**CPP4**			**MDV1**			**G377B 3D7**			**G377B PfKE04**		
	Estimate	**95% CI**	***p* value**	**Estimate**	**95% CI**	***p* value**	**Estimate**	**95% CI**	***p* value**	**Estimate**	**95% CI**	***p* value**
Age Group												
0 - 5 years	0.00	.	.	0.00	.	.	0.00	.	.	0.00	.	.
6 - 10 years	0.08	-0.02, 0.17	0.1297	0.10	0, 0.21	0.0579	0.20	0.09, 0.31	**0.0002**	0.21	0.11, 0.32	**0.0001**
11 - 15 years	-0.01	-0.14, 0.12	0.8783	0.12	-0.12, 0.37	0.3194	0.19	0.03, 0.35	**0.0173**	0.24	0.08, 0.39	**0.0033**

Covariate	CPP4			MDV1			G377B 3D7			G377B PfKE04		
	Estimate	**95% CI**	***p* value**	**Estimate**	**95% CI**	***p* value**	**Estimate**	**95% CI**	***p* value**	**Estimate**	**95% CI**	***p* value**
Asexual parasite positive	0.19	0.07, 0.31	**0.0014**	0.20	0.07, 0.34	**0.0033**	0.27	0.15, 0.39	**<0.001**	0.25	0.13, 0.37	**<0.001**
Gametocyte positive	0.15	0.01, 0.28	**0.0318**	0.16	-0.01, 0.32	0.0592	0.1	-0.02, 0.23	0.1091	0.09	-0.03, 0.22	0.1516
Sickle												
Normal	0.00	.	.	0.00	.	.	0.00	.	.	0.00	.	.
Heterozygous	-0.02	-0.14, 0.09	0.7038	-0.11	-0.23, 0.01	0.0689	0	-0.14, 0.13	0.9554	0.01	-0.13, 0.15	0.8872
α-thalassaemia												
Normal	0.00	.	.	0.00	.	.	0.00	.	.	0.00	.	.
Heterozygous	0.00	-0.1, 0.09	0.9236	0.01	-0.11, 0.13	0.8471	0.04	-0.07, 0.15	0.4963	0.01	-0.09, 0.11	0.8500
Homozygous	-0.04	-0.18, 0.09	0.5056	-0.1	-0.24, 0.03	0.1315	-0.03	-0.2, 0.14	0.7373	-0.07	-0.25, 0.11	0.4667
Subcohort												
Junju	0.00	.	.	0.00	.	.	0.00	.	.	0.00	.	.
Ngerenya early	-0.12	-0.28, 0.04	0.1352	-0.02	-0.23, 0.19	0.8785	-0.11	-0.27, 0.06	0.1978	-0.11	-0.26, 0.05	0.1739
Ngerenya late	0.01	-0.1, 0.12	0.8821	0	-0.12, 0.12	0.9528	-0.12	-0.23, -0.004	**0.0426**	-0.11	-0.22, 0.01	0.0669
Covariate	**PSOP25 3D7**			**PSOP25 PfKE04**			**AMA1**			**GE**		
	Estimate	**95% CI**	***p* value**	**Estimate**	**95% CI**	***p* value**	**Estimate**	**95% CI**	***p* value**	**Estimate**	**95% CI**	***p* value**
Age Group												
0 - 5 years	0.00	.	.	0.00	.	.	0.00	.	.	0.00	.	.
6 - 10 years	0.11	0, 0.22	0.0516	0.07	-0.06, 0.19	0.2848	0.39	0.13, 0.64	**0.0029**	0.11	-0.1, 0.32	0.3087
11 - 15 years	0.01	-0.12, 0.15	0.8324	0.12	-0.03, 0.27	0.1145	0.68	0.29, 1.07	**0.0006**	0.22	-0.08, 0.53	0.1478
Asexual parasite positive	0.09	-0.07, 0.25	0.2824	0.18	0.02, 0.34	**0.0284**	1.04	0.75, 1.32	**<0.001**	0.45	0.21, 0.68	**0.0002**
Gametocyte positive	0.12	-0.01, 0.25	0.0675	0.14	-0.01, 0.3	0.0737	0.46	0.12, 0.81	**0.0089**	0.28	0.02, 0.54	**0.0313**
Sickle												
Normal	0.00	.	.	0.00	.	.	0.00	.	.	0.00	.	.
Heterozygous	-0.01	-0.13, 0.11	0.8516	0.02	-0.11, 0.14	0.8042	-0.09	-0.41, 0.23	0.5899	0.13	-0.09, 0.36	0.2453

Covariate	PSOP25 3D7			PSOP25 PfKE04			AMA1			GE		
	Estimate	**95% CI**	***p* value**	**Estimate**	**95% CI**	***p* value**	**Estimate**	**95% CI**	***p* value**	**Estimate**	**95% CI**	***p* value**
α-thalassaemia												
Normal	0.00	.	.	0.00	.	.	0.00	.	.	0.00	.	.
Heterozygous	-0.08	-0.2, 0.04	0.2111	0.03	-0.11, 0.17	0.7010	-0.13	-0.4, 0.14	0.3335	-0.24	-0.45, -0.03	**0.0222**
Homozygous	-0.10	-0.23, 0.03	0.1155	-0.1	-0.25, 0.05	0.1784	-0.05	-0.4, 0.31	0.7999	-0.3	-0.57, -0.04	**0.0250**
Subcohort												
Junju	0.00	.	.	0.00	.	.	0.00	.	.	0.00	.	.
Ngerenya early	-0.16	-0.32, 0	0.0534	-0.14	-0.34, 0.06	0.1756	-0.02	-0.4, 0.36	0.9154	-0.20	-0.49, 0.1	0.1853
Ngerenya late	-0.03	-0.15, 0.1	0.6939	0.06	-0.06, 0.17	0.3531	-0.19	-0.48, 0.1	0.2054	-0.3	-0.54, -0.06	**0.0150**

P values in bold are statistically significant ($p<0.05$).

5.5.3.3. Factors associated with the magnitude of antibody response: AFIRM cohort

The AFIRM cohort provided an opportunity to analyse the relationship between season and immune responses to the gametocyte antigens. Additionally, as parasitaemia was measured by microscopy and by the highly sensitive nucleic acid sequence-based amplification (NASBA, *18S* for all parasites and female gametocyte specific *Pfs25* for gametocytes), it was possible to explore the effect of patent versus sub-patent parasitaemia. Results from the univariable analysis are provided in 9.9 **Appendix 9**.

In both the univariable multivariable analyses, increasing age was associated with higher antibody responses to Pfs230, AMA1 and GE (

Table 5.7). For G377B PfKE04, only the >15-year age group remained independently associated with higher antibody responses in the multivariable analysis. Older age (>15 years of age) was similarly associated with increased antibody responses to CVMPPP, PSOP1 and G377B 3D7. The >15-year age group was only associated with increased antibody responses to PEB-P in the multivariable analysis, indicating that the strength of this association may have been increased after adjusting for confounding factors or a non-causal relationship. Antibody responses to CPP4 were not associated with age.

Both univariable and multivariable analyses indicated that parasite positivity (either patent or sub-patent) was associated with increased responses to all the antigens except for Pfs230 or PSOP1. For Pfs230, sub-patent parasitaemia was not independently associated with the magnitude of antibody response after adjusting for age and gametocyte positivity. For CVMPPP, PEB-P, MDV1, G377B (both variants) and GE, both patent and sub-patent parasitaemia were associated with an increased magnitude of antibody responses. In an analysis limited to parasite positive individuals and including the same covariates as in the full models (**9.9 Appendix 9**), patent parasitaemia was not associated with higher antibody responses to any of the gametocyte antigens when compared to sub-patent parasitaemia. With sub-patent gametocyte positivity, gametocyte positivity was only an independent predictor of the magnitude of antibody response to Pfs230 after adjusting for age and asexual parasitaemia (estimate 0.23, 95% CI: 0.02 – 0.44, $p = 0.03$).

When I considered season, there was no evidence that it was independently predictive of antibody responses to any of the antigens. Conversely, the sickle AS genotype was associated with reduced antibody responses to PEB-P in both univariable and multivariable analysis (multivariable analysis: estimate -0.24, 95% CI: -0.42 – 0.05, $p = 0.01$). None of the gametocyte antigens was significantly associated with the α-thalassemia genotypes. As observed in the KMLRC, similar associations between increasing age and increased antibody responses as well as increased antibody responses with concurrent parasitaemia were observed for both G377B variants, with marginal differences in the R^2 values of models predicting the magnitude of antibody responses to either antigen (G377B 3D7 $R^2 = 0.34$, G377B 10668 $R^2 = 0.36$).

Table 5.7: Multivariable linear regression models of the factors predicting the magnitude of antibody response to the gametocyte antigens, AMA1 and gametocyte extract – AFIRM cohort

Covariate	Pfs230			CVMPPP			PEB-P			PSOP1		
	Estimate	**95% CI**	***p* value**	**Estimate**	**95% CI**	***p* value**	**Estimate**	**95% CI**	***p* value**	**Estimate**	**95% CI**	***p* value**
Age Group												
0 - 5 years	0.00	.	.	0.00	.	.	0.00	.	.	0.00	.	.
6 - 15 years	0.19	0.03, 0.34	**0.0172**	0.10	-0.09, 0.3	0.3032	-0.08	-0.25, 0.09	0.3566	0.07	-0.08, 0.22	0.3905
>15 years	0.56	0.4, 0.71	**<0.001**	0.26	0.07, 0.46	**0.0093**	0.18	0, 0.35	**0.0453**	0.50	0.35, 0.65	**<0.001**
Parasitaemia												
Parasite negative	0.00	.	.	0.00	.	.	0.00	.	.	0.00	.	.
Sub-patent only	0.07	-0.08, 0.22	0.3704	0.30	0.11, 0.49	**0.0028**	0.19	0.02, 0.36	**0.0300**	0.04	-0.11, 0.19	0.5671
Patent	-0.06	-0.34, 0.23	0.6936	0.35	0.01, 0.68	**0.0436**	0.36	0.07, 0.66	**0.0169**	0.20	-0.06, 0.46	0.1309
Gametocyte positive	0.23	0.02, 0.44	**0.0300**	0.11	-0.14, 0.37	0.3879	0.13	-0.09, 0.35	0.2403	0.07	-0.12, 0.26	0.4809
Sickle												
Normal	0.00	.	.	0.00	.	.	0.00	.	.	0.00	.	.
Heterozygous	0.13	-0.03, 0.3	0.1091	-0.18	-0.39, 0.03	0.0932	-0.24	-0.42, -0.05	**0.0126**	0.00	-0.16, 0.16	0.9899
α-thalassaemia												
Normal	0.00	.	.	0.00	.	.	0.00	.	.	0.00	.	.
Heterozygous	-0.08	-0.24, 0.07	0.2736	0.03	-0.15, 0.22	0.7362	0.12	-0.04, 0.28	0.1567	-0.01	-0.16, 0.13	0.8635
Homozygous	-0.06	-0.25, 0.13	0.5541	-0.07	-0.3, 0.17	0.5835	0.16	-0.04, 0.37	0.1187	0.11	-0.07, 0.29	0.2415
Season												
Dry	0.00	.	.	0.00	.	.	0.00	.	.	0.00	.	.
Wet	-0.11	-0.24, 0.02	0.0982	-0.12	-0.28, 0.04	0.1512	0.11	-0.03, 0.26	0.1189	0.06	-0.06, 0.19	0.3429

Covariate	CPP4			MDV1			G377B 3D7			G377B PfKE04		
	Estimate	**95% CI**	***p* value**	**Estimate**	**95% CI**	***p* value**	**Estimate**	**95% CI**	***p* value**	**Estimate**	**95% CI**	***p* value**
Age Group												
0 - 5 years	0.00	.	.	0.00	.	.	0.00	.	.	0.00	.	.
6 - 15 years	0.01	-0.14, 0.16	0.9012	0.15	0, 0.31	0.0574	0.16	0, 0.31	0.0500	0.16	-0.01, 0.32	0.0593
>15 years	0.08	-0.07, 0.23	0.2978	0.45	0.3, 0.61	**<0.001**	0.48	0.32, 0.63	**<0.001**	0.50	0.34, 0.66	**<0.001**
Parasitaemia												
Parasite negative	0.00	.	.	0.00	.	.	0.00	.	.	0.00	.	.
Sub-patent only	0.16	0.01, 0.31	**0.0387**	0.32	0.16, 0.48	**<0.001**	0.34	0.19, 0.49	**<0.001**	0.36	0.2, 0.52	**<0.001**
Patent	0.25	-0.01, 0.5	0.0633	0.40	0.12, 0.67	0.0047	0.29	0.03, 0.56	**0.0329**	0.34	0.06, 0.62	**0.0175**
Gametocyte positive	0.03	-0.16, 0.23	0.7528	0.08	-0.12, 0.29	0.4406	0.13	-0.07, 0.33	0.2144	0.16	-0.05, 0.37	0.1320
Sickle												
Normal	0.00	.	.	0.00	.	.	0.00	.	.	0.00	.	.
Heterozygous	-0.04	-0.21, 0.12	0.5930	-0.02	-0.19, 0.15	0.8289	-0.06	-0.23, 0.11	0.4973	-0.16	-0.33, 0.02	0.0817
α-thalassaemia												
Normal	0.00	.	.	0.00	.	.	0.00	.	.	0.00	.	.
Heterozygous	-0.01	-0.15, 0.14	0.9410	0.00	-0.15, 0.15	0.9735	0.02	-0.13, 0.16	0.8371	0.09	-0.07, 0.24	0.2661
Homozygous	0.03	-0.15, 0.21	0.7257	0.09	-0.09, 0.28	0.3253	-0.05	-0.24, 0.13	0.5879	0.02	-0.17, 0.22	0.8111
Season												
Dry	0.00	.	.	0.00	.	.	0.00	.	.	0.00	.	.
Wet	0.00	-0.13, 0.12	0.9864	0.00	-0.13, 0.13	0.9767	0.03	-0.1, 0.16	0.6370	-0.01	-0.14, 0.13	0.9332

Covariate	AMA1			GE		
	Estimate	**95% CI**	***p* value**	**Estimate**	**95% CI**	***p* value**
Age Group						
0 - 5 years	0.00	.	.	0.00	.	.
6 - 15 years	0.37	0.11, 0.63	**0.0065**	0.25	0, 0.49	**0.0472**
>15 years	0.82	0.55, 1.08	**<0.001**	0.85	0.6, 1.09	**<0.001**
Parasitaemia						
Parasite negative	0.00	.	.	0.00	.	.
Sub-patent only	0.26	0, 0.52	0.0514	0.44	0.2, 0.68	**<0.001**
Patent	0.56	0.1, 1.01	**0.0169**	0.56	0.15, 0.98	**0.0081**
Gametocyte positive	0.54	0.2, 0.88	**0.0023**	0.13	-0.17, 0.44	0.3968
Sickle						
Normal	0.00	.	.	0.00	.	.
Heterozygous	0.03	-0.25, 0.32	0.8266	-0.12	-0.38, 0.14	0.3782
α-thalassaemia						
Normal	0.00	.	.	0.00	.	.
Heterozygous	0.23	-0.02, 0.48	0.0770	-0.03	-0.26, 0.21	0.8213
Homozygous	0.33	0.02, 0.65	**0.0380**	0.16	-0.13, 0.45	0.2711
Season						
Dry	0.00	.	.	0.00	.	.
Wet	-0.02	-0.24, 0.2	0.8789	-0.02	-0.22, 0.19	0.8835

P values in bold are statistically significant ($p<0.05$).

5.5.3.4. Breadth of response analysis

The factors associated with the recognition of a greater number of antigens, which I termed the breadth of response, were also investigated. For this, I analysed each of the cohorts (KMLRC, AFIRM and LAMB) separately. For the KMLRC, only the adjusted analysis is interpreted as explained in section 5.5.3.2. From the multivariable analysis of the KMLRC cohort, (**Table 5.8**) increased age was associated with increased breadth of antigen recognition. Additionally, the 6 – 10-year age group was associated with increased breadth (estimate 0.10, 95% CI: 0.03 – 0.16, $p = 0.004$). Concurrent parasitaemia, whether asexual parasites (estimate 0.20, 95% CI: 0.13, 0.28, $p<0.0001$) or gametocytes (estimate 0.12, 95% CI: 0.03 – 0.21, $p = 0.002$) was also associated with an increased breadth of recognition. As for sickle and α-thalassaemia genotypes, there were no associations observed.

Similarly, for the AFIRM cohort, increasing age and concurrent parasitaemia (as detected by molecular methods) were associated with increased breadth (**9.9 Appendix 9**). For the LAMB cohort, concurrent parasitaemia (as detected by PCR) was not associated with the breadth of recognition in either univariable or multivariable analysis (**Table 5.9**). However, sampling timepoint was associated with breadth of antigen recognition. In the univariable analysis, there was decreased breadth associated with days 56 to 112. After adjusting for the other variables, only the association with day 86 and decreased breadth remained (estimate -0.06, 95% CI: -0.11, -0.02, $p = 0.008$).

Table 5.8: Linear regression analysis of the factors influencing the number of antigens recognised by the study participants – KMLRC cohort

Covariate	Univariable					Multivariable		
	Mean	**95% CI**	**Estimate**	**95% CI**	***p* value**	**Estimate**	**95% CI**	***p* value**
Age Group								
0 - 5 yrs	0.58	0.54, 0.63	0.00	.	.	0.00	.	.
6 - 10 yrs			0.10	0.03, 0.17	**0.0069**	0.10	0.03, 0.16	**0.0036**
11 - 15 yrs			0.11	-0.01, 0.23	0.0730	0.09	-0.02, 0.2	0.1084
Asexual parasite positive			0.26	0.2, 0.32	**<0.001**	0.20	0.13, 0.28	**<0.001**
Gametocyte positive			0.20	0.13, 0.27	**<0.001**	0.12	0.03, 0.21	**0.0073**
Sickle								
Normal	0.62	0.58, 0.66	0.00	.	.	0.00	.	.
Heterozygous			0.06	-0.03, 0.15	0.2001	0.02	-0.06, 0.1	0.6297
α - Thalassaemia								
Normal	0.67	0.6, 0.73	0.00	.	.	0.00	.	.
Heterozygous			-0.06	-0.14, 0.02	0.1555	-0.03	-0.1, 0.04	0.3869
Homozygous			-0.05	-0.16, 0.05	0.3144	-0.02	-0.12, 0.07	0.6236
Cohort								
Junju	0.70	0.65, 0.76	0.00	.	.	0.00	.	.
Ngerenya Early			0.01	-0.09, 0.11	0.8851	-0.03	-0.13, 0.08	0.6351
Ngerenya Late			-0.17	-0.24, -0.09	**<0.001**	-0.04	-0.12, 0.04	0.2991

* Mean of the number of antigens recognised (breadth, expressed as a proportion) for the reference group, with corresponding 95% confidence interval.
[a] Parasitaemia as determined by microscopy.
P values in bold are statistically significant ($p<0.05$).

Table 5.9: Linear regression analysis of the factors influencing the number of antigens recognised by the study participants – LAMB cohort

Covariate	Univariable					Multivariable		
	Mean	**95% CI**	**Estimate**	**95% CI**	***p* value**	**Estimate**	**95% CI**	***p* value**
Day								
Day 0	0.83	0.78, 0.89	0.00	.	.	0.00	.	.
Day 14			-0.03	-0.07, 0.02	0.1987	-0.03	-0.08, 0.02	0.2240
Day 28			-0.03	-0.08, 0.01	0.0909	-0.03	-0.08, 0.01	0.1414
Day 56			-0.06	-0.1, -0.02	**0.0034**	-0.04	-0.09, 0	0.0653
Day 84			-0.08	-0.12, -0.04	**0.0003**	-0.06	-0.11, -0.02	**0.0083**
Day 112			-0.06	-0.1, -0.02	**0.0047**	-0.04	-0.09, 0	0.0826
Parasite positive[a]			0.03	-0.01, 0.06	0.1317	0.02	-0.02, 0.06	0.2399
Gametocyte positive[a]			0.02	-0.02, 0.07	0.3285	0.00	-0.05, 0.05	0.8869
Sickle								
Normal	0.8	0.75, 0.85	0.00	.	.	0.00	.	.
Heterozygous			-0.04	-0.15, 0.07	0.4981	0.01	-0.11, 0.13	0.9268
α - Thalassaemia								
Normal	0.78	0.7, 0.85	0.00	.	.	0.00	.	.
Heterozygous			0.02	-0.08, 0.13	0.6384	0.06	-0.05, 0.17	0.3257
Homozygous			0.02	-0.13, 0.17	0.8228	0.02	-0.15, 0.19	0.8202
Anaemia								
No	0.79	0.74, 0.84	0.00	.	.	0.00	.	.
Yes			0.01	-0.03, 0.05	0.6205	0.00	-0.04, 0.04	0.9247
Multiplicity of Infection								
No	0.79	0.74, 0.84	0.00	.	.	0.00	.	.
Yes			0.02	-0.01, 0.05	0.2286	0.01	-0.03, 0.05	0.7236

* Mean of the number of antigens recognised (breadth, expressed as a proportion) for the reference group, with corresponding 95% confidence interval.

[a] Parasitaemia as determined by PCR (*18S* qPCR for all parasites and *Pfs25* qRT-PCR for female gametocytes).
P values in bold are statistically significant ($p<0.05$).

5.5.4. Dynamics of antibody responses over time – LAMB cohort

Little is known about how naturally acquired anti-gametocyte immunity changes over time, and how this relates to the various markers of malaria exposure. To better understand these dynamics, I analysed data from the LAMB cohort, a cohort of adults recruited and followed up for 112 days with regular sampling to detect parasites and the infectivity of participants.

5.5.4.1. Parasite density and prevalence over the follow-up period

Parasite detection was carried out by both microscopy and molecular detection;*18S* qPCR for all parasites and *Pfs25*-female-specific qRT-PCR for gametocytes. Parasite densities appeared stable with no significant variation over the follow-up period when detection was by qPCR ($p = 0.75$) (**Figure 5.8 (A)**). Similarly, when detection was by microscopy (**Figure 5.8 (B)**), asexual parasite densities did not vary significantly over the follow-up period ($p = 0.54$). As for gametocyte densities, there was no clear trend observed. Though densities appeared stable between day 0 and day 28 (qRT-PCR detection, $p = 0.86$), few individuals were positive at the later time points (**Figure 5.8 (C)**). Only one individual had microscopically detectable gametocytes throughout follow-up.

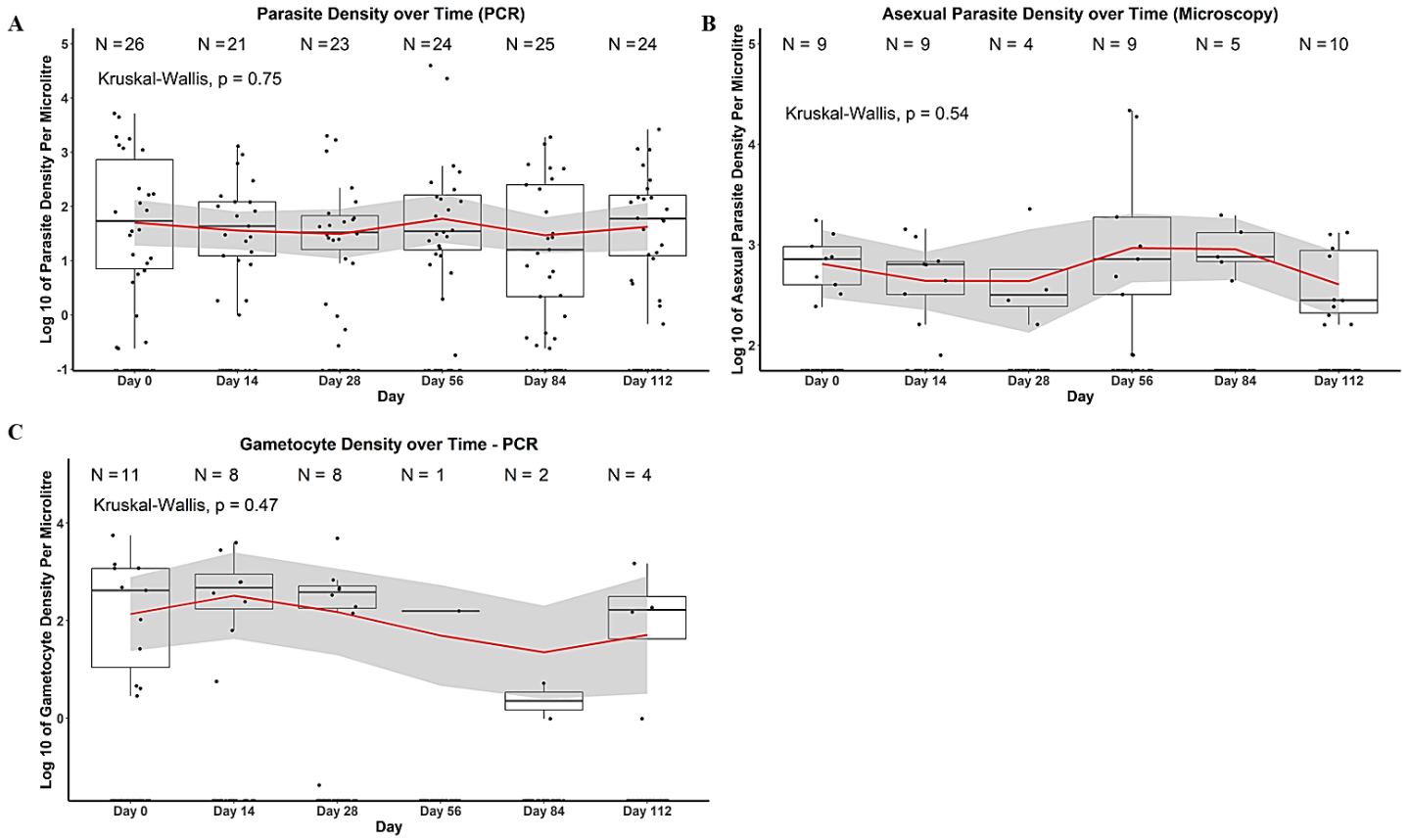


Figure 5.8: Parasite density over 112 days of follow-up in the LAMB cohort. (A) Parasite density over follow-up period as detected by *18S* qPCR. (B) Asexual parasite density over follow-up period as detected by microscopy. (C) Gametocyte density over the follow-up period as detected by *Pfs25* qRT-PCR (detects female gametocytes). Kruskal-Wallis tests were used to compare parasite densities at the different timepoints, number of parasite positive individuals (N) is included at the top of each graph for each timepoint. Trend lines shown were estimated using LOESS smoothing, with the red line indicating the estimated mean and the shaded grey areas the 95% confidence intervals. The boxes of boxplots display the median bound by the first and third quartiles, with the whiskers depicting the lowest and highest values (excluding outliers). The dots indicate individual datapoints.

5.5.4.2. Factors associated with the magnitude of antibody response to the gametocyte antigens

In addition to looking at sampling timepoint (day), I also explored how the variables specified in **5.5.3** above impact the magnitude of antibody responses to the gametocyte antigens over time. Additionally, as information on haemoglobin levels and MOI were available, and may be associated with gametocyte carriage[77,228,564], I investigated these factors as well. I included day as a categorical variable to better assess how antibody responses varied at each of the time points using day 0 as the reference category. Results from the univariable analysis are provided in **9.9 Appendix 9**, while the results from multivariable analyses are presented here (**Table 5.10**).

There was no evidence for a decline in the magnitude of antibody response over the follow-up period for G377B 3D7, PSOP25 (both variants) and AMA1. Additionally, there was no longer evidence for a decline in antibody responses to CVMPPP, MDV1 and PSOP1 after adjusting for parasite prevalence. For Pfs230 and CPP4, there was a fluctuation in responses over time with decreases in the magnitude of antibody response at day 28 for Pfs230 (estimate -0.14, 95% CI: -0.25, -0.02, $p = 0.02$) and day 56 for CPP4 (estimate -0.13, 95% CI: -0.24, -0.01, $p = 0.03$). While in the univariable analysis there was an indication for a steady decline in the magnitude of response associated with days 28 up to 112 for PEB-P and GE, only day 112 remained associated with a decrease in antibody responses to PEB-P and day 28 for GE (estimate -0.14, 95% CI: -0.23, -0.06, $p = 0.001$ and estimate -0.05, 95% CI: -0.1, -0.001, $p = 0.049$ respectively) after multivariable analysis. Additionally, day 112 was now also associated with a decrease in the magnitude of responses to G77B PfKE04 (estimate -0.10, 95% CI: -0.18, -0.02, $p = 0.02$). Gametocyte prevalence fluctuated significantly over the follow-up period (**Table 5.4**), which may relate to the fluctuation seen in antibody levels for some gametocyte antigens.

Parasitaemia was not an independent predictor of antibody responses to any antigens after adjusting for the other variables. Likewise, sickle cell trait was not associated with antibody responses to any of the antigens. However, associations were seen with α-thalassemia. Homozygous individuals were associated with increased antibody

responses to MDV1 (estimate 0.48, 95% CI: 0.17, 0.79, $p = 0.004$) and G377B 3D7 (estimate 0.43, 95% CI: 0.03, 0.82, $p = 0.04$). Anaemia was associated with increased antibody responses to PEB-P (estimate 0.10, 95% CI: 0.02, 0.17, $p = 0.02$), with G377B 3D7, AMA1 and PEB-P no longer associated after adjusting for day and parasite positivity. For MOI, it was not an independent predictor of the magnitude of antibody responses to any of the antigens.

While being homozygous for α-thalassaemia was associated with an increased magnitude of response to both G377B variants, there was a difference in the associations with sampling timepoint. The magnitude of antibody response to G377B 3D7 variant was stable over time, while day 112 was associated with decreased antibody responses to G377B PfKE04. Differences in the R^2 values of models predicting the magnitude of antibody responses to either antigen were marginal (G377B 3D7 $R^2 = 0.18$, G377B PfKE04 $R^2 = 0.17$). There were no associations between any of the variables investigated and the magnitude of antibody response to either of the PSOP25 variants. Additionally, differences in the R^2 values of models predicting the magnitude of antibody responses to either antigen were marginal (PSOP25 3D7 $R^2 = 0.08$, PSOP25 PfKE04 $R^2 = 0.03$).

Table 5.10: Multivariable linear regression analysis (mixed effects model) of the factors predicting the magnitude of antibody response to the gametocyte antigens, AMA1 and gametocyte extract.

Covariate	Pfs230			CVMPPP			PEB-P			PSOP1		
	Estimate	**95% CI**	***p* value**	**Estimate**	**95% CI**	***p* value**	**Estimate**	**95% CI**	***p* value**	**Estimate**	**95% CI**	***p* value**
Day												
Day 0	0.00	.	.	0.00	.	.	0.00	.	.	0.00	.	.
Day 14	-0.03	-0.16, 0.09	0.5941	-0.07	-0.21, 0.07	0.3081	-0.02	-0.11, 0.07	0.6670	0.05	-0.08, 0.18	0.4710
Day 28	-0.14	-0.25, -0.02	**0.0176**	-0.11	-0.23, 0.01	0.0843	-0.03	-0.11, 0.05	0.5033	0.03	-0.09, 0.14	0.6606
Day 56	-0.08	-0.2, 0.04	0.1708	-0.07	-0.21, 0.06	0.2700	-0.03	-0.12, 0.05	0.4292	-0.02	-0.14, 0.11	0.7940
Day 84	-0.11	-0.23, 0	0.0616	-0.09	-0.22, 0.03	0.1481	-0.08	-0.16, 0	0.0564	-0.03	-0.15, 0.09	0.6548
Day 112	-0.04	-0.16, 0.08	0.4901	-0.06	-0.19, 0.07	0.3504	-0.14	-0.23, -0.06	**0.0014**	-0.06	-0.19, 0.06	0.3068
Parasitaemia												
Parasite negative	0.00	.	.	0.00	.	.	0.00	.	.	0.00	.	.
Sub-patent only	0.03	-0.07, 0.14	0.5245	0.02	-0.09, 0.13	0.7042	0.05	-0.02, 0.13	0.1563	0.01	-0.09, 0.12	0.7990
Patent	0.00	-0.12, 0.13	0.9646	-0.02	-0.16, 0.12	0.7851	0.01	-0.08, 0.11	0.7739	0.07	-0.07, 0.2	0.3370
Gametocyte positive	0.04	-0.09, 0.17	0.5607	0.10	-0.05, 0.24	0.2020	0.08	-0.01, 0.18	0.0922	0.09	-0.05, 0.23	0.2165
Sickle												
Normal	0.00	.	.	0.00	.	.	0.00	.	.	0.00	.	.
Heterozygous	-0.07	-0.32, 0.18	0.5916	-0.26	-0.61, 0.09	0.1535	-0.09	-0.38, 0.21	0.5751	0.18	-0.1, 0.46	0.2059
α-thalassaemia												
Normal	0.00	.	.	0.00	.	.	0.00	.	.	0.00	.	.
Heterozygous	0.02	-0.21, 0.26	0.8629	0.29	-0.04, 0.61	0.0898	0.01	-0.27, 0.28	0.9598	0.19	-0.07, 0.45	0.1687
Homozygous	0.18	-0.17, 0.53	0.3254	-0.13	-0.61, 0.36	0.6158	-0.09	-0.5, 0.32	0.6739	-0.03	-0.42, 0.36	0.8969
Anaemia												
No	0.00	.	.	0.00	.	.	0.00	.	.	0.00	.	.
Yes	0.05	-0.06, 0.15	0.3688	0.04	-0.08, 0.15	0.5213	0.10	0.02, 0.17	**0.0159**	0.02	-0.09, 0.13	0.7460

Covariate	Pfs230			CVMPPP			PEB-P			PSOP1		
	Estimate	**95% CI**	***p* value**	**Estimate**	**95% CI**	***p* value**	**Estimate**	**95% CI**	***p* value**	**Estimate**	**95% CI**	***p* value**
MOI												
No	0.00	.	.	0.00	.	.	0.00	.	.	0.00	.	.
Yes	0.03	-0.06, 0.13	0.4964	0.09	-0.02, 0.2	0.1000	-0.04	-0.11, 0.04	0.3291	0.04	-0.06, 0.14	0.4447
Covariate	**CPP4**			**MDV1**			**G377B 3D7**			**G377B PfKE04**		
	Estimate	**95% CI**	***p* value**	**Estimate**	**95% CI**	***p* value**	**Estimate**	**95% CI**	***p* value**	**Estimate**	**95% CI**	***p* value**
Day												
Day 0	0.00	.	.	0.00	.	.	0.00	.	.	0.00	.	.
Day 14	-0.03	-0.15, 0.08	0.5744	0.01	-0.09, 0.12	0.7869	-0.01	-0.17, 0.14	0.8534	-0.01	-0.1, 0.07	0.7428
Day 28	-0.05	-0.15, 0.06	0.3741	-0.07	-0.16, 0.02	0.1439	-0.08	-0.21, 0.05	0.2432	0.01	-0.07, 0.08	0.8575
Day 56	-0.13	-0.24, -0.01	**0.0280**	-0.02	-0.12, 0.08	0.7321	-0.01	-0.16, 0.13	0.8420	-0.03	-0.11, 0.06	0.5247
Day 84	-0.05	-0.16, 0.06	0.3464	-0.05	-0.15, 0.05	0.3133	-0.06	-0.2, 0.08	0.4155	-0.05	-0.13, 0.03	0.1882
Day 112	-0.02	-0.13, 0.09	0.7268	-0.02	-0.11, 0.08	0.7391	-0.04	-0.18, 0.1	0.5983	-0.10	-0.18, -0.02	**0.0170**
Parasitaemia												
Parasite negative	0.00	.	.	0.00	.	.	0.00	.	.	0.00	.	.
Sub-patent only	0.03	-0.06, 0.13	0.5094	0.08	-0.01, 0.16	0.0790	0.01	-0.11, 0.13	0.8684	-0.01	-0.08, 0.06	0.7582
Patent	0.00	-0.12, 0.12	0.9859	0.09	-0.01, 0.2	0.0920	-0.14	-0.29, 0.02	0.0839	-0.04	-0.13, 0.05	0.3966
Gametocyte positive	0.03	-0.09, 0.16	0.6168	0.11	-0.01, 0.22	0.0678	0.11	-0.05, 0.27	0.1637	0.03	-0.06, 0.12	0.5053
Sickle												
Normal	0.00	.	.	0.00	.	.	0.00	.	.	0.00	.	.
Heterozygous	-0.06	-0.32, 0.2	0.6562	-0.01	-0.3, 0.28	0.9408	-0.03	-0.33, 0.27	0.8315	-0.07	-0.34, 0.2	0.5927
α-thalassaemia												
Normal	0.00	.	.	0.00	.	.	0.00	.	.	0.00	.	.
Heterozygous	0.14	-0.1, 0.38	0.2658	0.14	-0.13, 0.41	0.3072	0.26	-0.02, 0.54	0.0753	0.18	-0.07, 0.43	0.1750
Homozygous	-0.05	-0.41, 0.32	0.8047	0.52	0.13, 0.92	**0.0135**	0.58	0.16, 1	**0.0092**	0.43	0.06, 0.8	**0.0294**
Anaemia												
No	0.00	.	.	0.00	.	.	0.00	.	.	0.00	.	.
Yes	0.01	-0.09, 0.11	0.8170	0.03	-0.06, 0.12	0.5432	0.08	-0.04, 0.21	0.2041	0.05	-0.03, 0.12	0.2083

Covariate	CPP4			MDV1			G377B 3D7			G377B PfKE04		
	Estimate	**95% CI**	***p* value**	**Estimate**	**95% CI**	***p* value**	**Estimate**	**95% CI**	***p* value**	**Estimate**	**95% CI**	***p* value**
MOI												
No	0.00	.	.	0.00	.	.	0.00	.	.	0.00	.	.
Yes	-0.03	-0.12, 0.07	0.5893	0.05	-0.04, 0.13	0.2650	0.02	-0.1, 0.14	0.7610	-0.05	-0.12, 0.02	0.1555
Covariate	**PSOP25 3D7**			**PSOP25 PfKE04**			**AMA1**			**GE**		
	Estimate	**95% CI**	***p* value**	**Estimate**	**95% CI**	***p* value**	**Estimate**	**95% CI**	***p* value**	**Estimate**	**95% CI**	***p* value**
Day												
Day 0	0.00	.	.	0.00	.	.	0.00	.	.	0.00	.	.
Day 14	0.07	-0.05, 0.2	0.2384	0.05	-0.03, 0.12	0.2387	0.10	-0.04, 0.24	0.1814	-0.01	-0.07, 0.05	0.8478
Day 28	-0.03	-0.14, 0.08	0.5658	-0.02	-0.08, 0.05	0.6224	-0.05	-0.17, 0.08	0.4862	-0.05	-0.1, -0.001	**0.0493**
Day 56	-0.06	-0.17, 0.06	0.3352	0.01	-0.06, 0.08	0.7811	0.04	-0.1, 0.17	0.5879	-0.03	-0.09, 0.02	0.2541
Day 84	-0.09	-0.2, 0.03	0.1321	-0.03	-0.1, 0.04	0.3833	0.01	-0.12, 0.14	0.8750	-0.05	-0.1, 0.01	0.0848
Day 112	0.02	-0.1, 0.13	0.7675	-0.03	-0.1, 0.04	0.3919	0.20	0.07, 0.34	**0.0036**	-0.05	-0.11, 0.002	0.0616
Parasitaemia												
Parasite negative	0.00	.	.	0.00	.	.	0.00	.	.	0.00	.	.
Sub-patent only	0.03	-0.07, 0.13	0.5670	0.00	-0.07, 0.06	0.8839	-0.01	-0.13, 0.11	0.8438	0.02	-0.03, 0.07	0.4808
Patent	-0.04	-0.17, 0.08	0.4974	0.04	-0.03, 0.12	0.2800	-0.06	-0.2, 0.09	0.4468	0.03	-0.03, 0.09	0.2823
Gametocyte positive	-0.01	-0.13, 0.12	0.9331	0.02	-0.06, 0.1	0.5952	0.07	-0.09, 0.23	0.3780	0.04	-0.02, 0.11	0.1681
Sickle												
Normal	0.00	.	.	0.00	.	.	0.00	.	.	0.00	.	.
Heterozygous	0.01	-0.21, 0.24	0.9157	-0.01	-0.23, 0.22	0.9421	-0.10	-0.6, 0.41	0.7100	-0.02	-0.4, 0.35	0.8985
α-thalassaemia												
Normal	0.00	.	.	0.00	.	.	0.00	.	.	0.00	.	.
Heterozygous	0.20	-0.01, 0.41	0.0698	0.11	-0.1, 0.31	0.3156	0.01	-0.46, 0.48	0.9619	0.18	-0.17, 0.52	0.3205
Homozygous	0.09	-0.23, 0.4	0.5978	0.21	-0.1, 0.52	0.1961	0.11	-0.63, 0.84	0.7765	0.01	-0.5, 0.52	0.9695
Anaemia												
No	0.00	.	.	0.00	.	.	0.00	.	.	0.00	.	.
Yes	0.04	-0.06, 0.15	0.3909	0.00	-0.06, 0.06	0.9947	0.03	-0.09, 0.15	0.6363	0.03	-0.02, 0.08	0.2212

Covariate	PSOP25 3D7			PSOP25 PfKE04			AMA1			GE		
	Estimate	**95% CI**	***p* value**	**Estimate**	**95% CI**	***p* value**	**Estimate**	**95% CI**	***p* value**	**Estimate**	**95% CI**	***p* value**
MOI												
No	0.00	.	.	0.00	.	.	0.00	.	.	0.00	.	.
Yes	0.07	-0.03, 0.16	0.1734	-0.03	-0.09, 0.03	0.3039	-0.07	-0.19, 0.04	0.2231	-0.01	-0.06, 0.04	0.7168

P values in bold are statistically significant ($p<0.05$).

5.5.5. Factors influencing infectiousness to mosquitoes

Direct membrane feeding assays were carried out using autologous sera from study participants of the AFIRM and LAMB cohorts to determine infectiousness to mosquitoes. Only six individuals were infectious to mosquitoes in both cohorts; hence power is limited for this analysis (**9.9 Appendix 9)**. I limited the analysis to participants with gametocytaemia, as detected by molecular methods, on the basis that this is the group most likely to transmit to mosquitoes[379,474,565]. I chose to evaluate age and parasite positivity in relation to infectiousness as they significant associations with antibody levels in the prior analyses. I tabulated the frequency of infectiousness by age and parasite status for both AFIRM and LAMB cohorts and carried out a fisher's exact test to test for significant differences in the categories. The influence of patent asexual parasitaemia compared to sub-patent parasitaemia was also included in the analysis. From the analysis, there was no evidence to suggest an influence of parasite status (**Table 5.11**) or age group (**Table 5.12**) on infectiousness for the AFIRM cohort ($p = 0.15$ and $p = 0.30$) respectively). Similarly, there was no influence of parasite status for the LAMB cohort ($p = 1$) (**Table 5.13**).

Table 5.11: 3 x 2 tables of the frequency of infectiousness by parasite status in the AFIRM cohort

	Infectiousness	
Parasite Status	**No**	**Yes**
Asexual Parasite Negative	1	0
Sub-patent parasitaemia	28	0
Patent parasitaemia	22	3
Total	51	3

Table 5.12: 3 x 2 tables of the frequency of infectiousness by age-group in the AFIRM cohort

	Infectiousness	
Age Group	**No**	**Yes**
0 - 5 years	15	2
6 - 15 years	18	1
>15 years	18	0
Age Group	**No**	**Yes**
Total	51	3

Table 5.13: 3 x 2 tables of the frequency of infectiousness by parasite status in the LAMB cohort

	Infectiousness	
Parasite Status	**No**	**Yes**
Asexual Parasite Negative	5	0
Sub-patent parasitaemia	14	2
Patent parasitaemia	11	1
Total	30	3

5.6.Discussion

In order to improve our understanding of naturally acquired immune responses to sexual stage antigens, I carried out seroepidemiological analyses using as targets the gametocyte proteins produced in **Chapter 4 (Table 4.5)** and sera from three cohorts of malaria-exposed individuals. Combining insights from **Chapter 2** and **Chapter 3**, I explored the relationship between key markers of malaria exposure as well as predictive indicators of gametocyte carriage to describe the factors associated with immune responses to sexual stage antigens. The targeted gametocyte proteins were CVMPPP, PEB-P, PSOP1, CPP4, MDV1, G377 (domain B, G377B), and PSOP25. Additionally, I included Pfs230 which has been extensively characterised in the context of anti-gametocyte immunity[74,200,203,218,415], and AMA1, which has been widely studied in the context of immune responses to asexual stages[469,549–552]. I also analysed immune responses to a prepared extract from a culture of mature gametocytes (GE) to evaluate its utility as a marker of recent exposure to gametocytes.

To determine whether the gametocyte antigens are antibody targets, I calculated the seroprevalence to each antigen using data from the AFIRM cohort. Antibody responses to MDV1, G377B (3D7 and PfKE04 variants) and GE were highly prevalent (>60%), as were responses to AMA1 as previously described[549,551,552]. Interestingly, though PSOP1 is hypothesised to be expressed predominantly at the ookinete stage,[566] it was recognised by the immune sera. Possibly, though expression may peak at the ookinete stage, it may begin much earlier. A previous study on the *P. berghei* proteome did detect the high expression of the PSOP1 homologue (PBANKA_0619200) in gametocytes[567], demonstrating a lack of exclusivity to the ookinete stage. For Pfs230, the estimated seroprevalence was 42.6%. Based on previous studies of seroprevalence to Pfs230 [214,227,415,568], estimates have been shown to vary widely depending on the study design and assay protocol employed (**Chapter 3**). Therefore, direct comparison of Pfs230 seroprevalence from this study with that from other studies is challenging.

There has been debate as to whether seroprevalence to gametocyte antigens increases with age, based on studies to Pfs230 and Pfs48/45. Some studies describe no increase with age [202,217,218,423], and others indicate an increase in seroprevalence with age[200,203,222,568]. In the AFIRM cohort, an increase in seroprevalence with age for all

the antigens (except CPP4) was observed. This supports the argument that individuals living in malaria-endemic areas do develop some level of long-lived antibody responses to sexual stage antigens. Additionally, seroprevalence to gametocyte antigens has been shown in some studies to be boosted during the malaria transmission season[200,217,227]. Within the AFIRM cohort, there was no general trend towards higher seroprevalence in the wet season among the antigens tested. Though parasite densities were higher in the wet season (**Figure 5.7**), there was substantial submicroscopic parasite carriage in the dry season (**Table 5.3**). Chronic submicroscopic gametocyte exposure has been described to boost anti-gametocyte antibodies[222] and could be responsible for sustaining antibody responses in the dry season.

In addition to seroprevalence, I also investigated factors that influence the magnitude of response to the gametocyte antigens using data from the three cohorts in separate multivariable analyses conducted using linear regression models. As with seroprevalence, there was an association between increasing age and increased antibody responses to the gametocyte antigens in the KMLRC and AFIRM cohorts. This was particularly evident for Pfs230 and G377 (both variants) where gradual increases in the magnitude of antibody responses with age were observed for both the KMLRC and AFIRM cohorts. A similar age-dependent increase was described by Stone *et al.* (2018) in their study, not only for Pfs230 and Pfs48/45 but also for a panel of novel antigens they thought to be important for NA-TBI[74].

Further, concurrent parasitaemia (asexual parasitaemia for the KMLRC cohort) was associated with increased antibody responses to the antigens in the KMLRC and AFIRM cohorts. Asexual parasitaemia is a strong predictor of gametocyte carriage[217,459,465] (also see **Chapter 3,** section **3.6**), which could in turn influence antibody responses to sexual stage antigens. Notably, in a sub-analysis of patent vs sub-patent parasitaemia for the AFIRM cohort, both patent and sub-patent parasitaemia were associated with higher antibody responses to CVMPPP, PEB-P, MDV1, G377B (both variants) and GE, in comparison to parasite negative individuals. However, when limited to parasite positive individuals, patent parasitaemia was not associated with higher responses to these antigens in comparison to sub-patent parasitaemia. This suggests that both patent and sub-patent parasite densities are equally important for boosting antibody responses to sexual stage antigens. There was no strong evidence to indicate that transmission intensity was an

independent predictor of the magnitude of antibody response to the gametocyte antigens in adjusted analysis. Due to challenges with proper matching of cases and controls in the KMLRC, this finding would require further verification in future analysis.

In multivariable analyses, gametocyte carriage was not always a strong independent predictor of antibody responses to the gametocyte antigens. In the KMLRC, microscopically determined gametocytaemia was associated with increased magnitude of antibody responses to GE, CVMPPP, PEB-P, and CPP4. However, in the AFIRM cohort (with gametocyte detection by molecular methods), gametocyte carriage did not independently predict gametocyte carriage. Though biologically plausible, the relationship between concurrent gametocytaemia and sexual stage responses may be confounded by 1) pre-existing responses from prior gametocyte exposure that persists after gametocyte clearance or suppression to submicroscopic levels, or 2) the lag between gametocyte exposure and acquisition of responses, relating to the time it takes to clear the circulating gametocytes for antigen presentation to the immune system[203,217,218,222,569]. Nonetheless, an association between patent gametocytaemia and increased anti-gametocyte responses was indicated in the KMLRC cohort for the gametocyte antigens CPP4, CVMPPP, and PEB-P as well as for GE. This could serve as evidence for a role for these antigens as markers of recent exposure to high-density gametocytaemia. Further investigation in larger, independent cohorts would be required to confirm these findings. Additionally, as GE is prepared from a gametocyte culture rather than recombinant protein, it could provide a more convenient means of screening individuals for gametocyte exposure.

Interestingly, in the sub-analysis of patent vs sub-patent parasitaemia in the AFIRM cohort, for Pfs230, gametocyte carriage (which was almost exclusively sub-patent, **Table 5.3)** was an even stronger predictor of antibody responses than asexual parasitaemia. A potential explanation for this might be that sub-patent gametocyte carriage could be a marker of individuals with chronic exposure to low-density parasite infections who then experience continuous boosting of responses[200,570]. This may allow the development of more long-lived stable antibody responses[217], which may be a better predictor of responses to Pfs230 in comparison to patent parasitaemia.

In addition to parasitaemia, haemoglobinopathies have also been shown in some studies to increase gametocyte carriage[79,442], which I hypothesised would result in increased antibody responses to the gametocyte antigens. What I found for sickle cell trait in the AFIRM cohort, was that for PEB-P, there was a modest association between sickle cell trait and reduced antibody responses. Individuals with sickle cell trait are protected from severe malaria and are less likely to experience the pathologies associated with clinical malaria[128,130,133], experiencing lower-density chronic infections instead[79]. Therefore, if indeed PEB-P is a marker of recent higher density parasitaemia, then this may explain the inverse relationship seen with sickle cell trait. This was the only significant observation observed across the three cohorts, and therefore further investigation to ascertain this finding is required. Varied associations between the different gametocyte antigens and α-thalassaemia were observed. This made it challenging to define the relationship between α-thalassaemia and the magnitude of anti-gametocyte antibody responses.

Season did not appear to be a strong predictor of antibody responses to the gametocyte antigens. Increases in responses to Pfs230 and Pfs48/45 in the rainy season have been described[200,217,227], presumably as a result of increased parasite prevalence and density. Parasite transmission occurs all year round at the Kenyan coast, though increased transmission is observed following the onset of the rainy season. This is evidenced by the fact that though parasite densities did increase in the rainy season (**Figure 5.7**), parasite prevalence as detected by molecular methods was similar in the two seasons (**Table 5.3**). Sampling timepoint in the rainy season may also affect the magnitude of response seen. By sampling at the onset, the peak and after the rainy season, Ouedraogo *et al.* (2011) showed that antibody responses to Pfs230 were highest at the peak of the rainy season but returned to initial levels by the end of the rainy season[217]. Sampling at such stated intervals over the rainy season instead of continuous sampling over the rainy season may provide a better approach to detecting an influence of season on boosting antibody responses to the gametocyte antigens.

The LAMB cohort provided an opportunity to investigate the dynamics, and potentially the longevity, of immune responses to the gametocyte antigens over time. For AMA1, antibody responses appeared stable over the four months of follow-up, as reported in the literature[550,552]. Similarly, antibody responses to the gametocyte antigens CVMPPP, MDV1, PSOP1, G377B 3D7 and both variants of PSOP25

appeared stable over time, with the other antigens showing varied fluctuations over the different timepoints. The Longevity of anti-gametocyte immunity has not been widely studied as most studies have been cross-sectional, or longitudinal with long sampling intervals (**Chapter 2**). Two studies have directly estimated the longevity of antibody responses to Pfs230 and Pfs48/45. Both described a short half-life, with Bousema *et al*. (2010) estimating an antibody half-life of 92 and 83 days respectively[222], while Ouedraogo *et al*. (2018) estimating a half-life of roughly a month[200]. Determination of half-life was by different methods with Ouedraogo *et al*. (2018)[200] also accounting for parasite exposure in addition to time, and this may explain the shorter half-life reported. Further investigation on a larger sample set, over a longer follow-up period, across all age groups, and with regular sampling is warranted.

. Anaemia and MOI have previously been associated with increased gametocyte carriage[77,228,564], which could lead to an association with increased immune responses to the gametocyte antigens. In the LAMB cohort, though there was an indication of increased immune responses to the PEB-P in individuals with anaemia, no association was seen with MOI. An association with anaemia could further serve to suggest an association for PEB-P with gametocyte carriage. As none of the previous associations between parasite prevalence was observed in the LAMB cohort, it may well be that small sample size, and an all adult cohort, do not provide the best means to disentangle factors predictive of anti-gametocyte immunity.

In addition to examining factors influencing antibody responses to individual antigens, I also looked at the breadth of response. Increasing age, gametocyte positivity and asexual parasite positivity were associated with the recognition of a higher number of antigens in both the KMLRC and AFIRM cohorts, and this has previously been described[227,568]. Moreover, as data on participant infectivity to mosquitoes was available for the AFIRM and LAMB cohorts, I hoped to investigate how anti-gametocyte antibody responses impacted infectiousness. Unfortunately, few individuals were infectious, limiting the power to carry out robust analyses.

For two of the antigens included in this study, variants based on 3D7 and a field isolate (PfKE04) were tested. Extensive polymorphism in vaccine candidates can impact vaccine efficacy in the field owing to the induction of variant-specific

responses[236,254,256,571]; therefore, this is an essential factor to consider. Though gametocyte antigens are relatively conserved[572], sequence variation does exist for some of the antigens. Acquah *et al*. (2017) studied the effects of variation in the 6C region of Pfs48/45 (a single non-synonymous SNP) and C0 region of Pfs230 (nine base pair deletion) on antibody responses [425]. They found no impact of these variations on antibody responses to the two antigens.

In this study, seroprevalence did not differ between variants of G377 in the AFIRM cohort. However, in the analysis of the factors associated with the magnitude of response to the antigens, associations were not always similar between variants of G377B and PSOP25. Nevertheless, associations with increasing age and concurrent parasitaemia and increased magnitude of antibody response were observed to both G377B variants in the KMLRC and AFIRM cohorts. To assess whether there are variant-specific differences in antibody responses, it will be necessary to carry out competition ELISAs using immune sera raised against each variant antigen. Owing to limited protein quantities and time constraints of the PhD, it was not possible to conduct competition ELISAs.

5.6.1. Limitations

The three cohorts allowed independent evaluation of the ability of some of the factors relating to malaria exposure to predict responses to the gametocyte antigens. However, not all variables were tested for each of the cohorts (e.g. unavailability of data on submicroscopic parasite carriage and infectivity in the KMLRC cohort), or they were not available due to study design (e.g. transmission intensity for AFIRM and season for KMLRC). Therefore, in cases where associations seemed unclear, (lack of clear trend with season) or where larger numbers would have possibly generated more robust evidence (analysis of infectiousness), a more extensive dataset would have been beneficial.

Additionally, only the LAMB cohort was longitudinal with follow-up carried out at relatively frequent intervals. However, the sample size was small and consisted only of adults making it challenging to ascertain and generalise the associations seen. Longitudinal studies with frequent sampling across all age groups will allow us to discern better the relationship between recent versus prior parasite exposure and sexual stage antibody kinetics[200,203], particularly if antibody responses are measured

both at the microscopic and submicroscopic level[217]. Such studies will also need to examine TBA at each of these time points to allow the impact on infectiousness to be evaluated[568].

For this study, I analysed a small panel of antigens which I endeavoured to produce as full-length ectodomains (refer to **Chapter 4**). While I was able to identify patterns of association as described in the literature, it is possible that naturally acquired sexual stage immune responses cannot be wholly elucidated from a subset of gametocyte antigens. Several studies have employed microarray platforms for the simultaneous detection of responses to hundreds of antigens[156,227,568], giving a possibly more representative picture of NAI to malaria. Indeed, it could very well be that breadth of response (or lack thereof) rather than responses to a small panel of antigens would be better prognostic indicators of individuals contributing disproportionately to the infectious reservoir.

That said, the utility of my approach is that it interrogates a smaller number of antigens that can be characterised more extensively in functional assays. I also evaluated the ability of a crude gametocyte extract (GE) to predict individuals with likely gametocyte exposure. Though GE seems to have some utility in predicting exposure to gametocytes, it could potentially contain shared antigens between asexual and sexual stages and hence may not be entirely gametocyte specific. Though care was taken to minimise the presence of asexual stage parasites and immature gametocytes, the gametocyte extract could contain some contamination from these stages. This could be mitigated against (at least for asexual stage antibody responses) by adjusting for responses to schizont lysate in future analysis. Unfortunately, owing to time constraints, I was unable to measure responses to schizont lysate for this study.

Finally, validation of the protein structure was not carried out, and therefore I cannot verify that the recombinant proteins produced assume their native conformation. Therefore, while the proteins do appear to be targets of naturally acquired antibody responses in this analysis, these results are preliminary and would require verification once information on the conformation of the recombinant protein is available.

5.6.2. Summary of overall findings

In summary, key factors that strongly associated with responses to the gametocyte antigens were age and concurrent parasitaemia. Notably, age-dependent acquisition

of responses to Pfs230 and boosting by sub-microscopic gametocyte densities may indicate that some level of immunological memory to gametocyte antigens. The association was only observed in a small subset of samples hence would warrant further investigation to exclude any impact of sample bias. Nevertheless, it highlights the importance of taking into account submicroscopic parasitaemia when describing the dynamics of naturally acquired responses to gametocyte antigens. Of the antigens tested, G377B seemed to share patterns of association with Pfs230 in comparison to the other antigens, and hence it would be interesting to see how immune sera raised against G377B performs in functional transmission-blocking assays.

PEB-P, CVMPPP, MDV1 and GE appeared to have potential as serological markers of recent gametocyte exposure. The evidence presented here warrants further evaluation of their prognostic ability. Few individuals were infectious in the cohorts studied; hence analyses were not well powered to explore features associated with infectiousness.

Chapter 6

Evaluation of identified gametocyte, gamete and ookinete antigens as candidate transmission-blocking vaccines

6.1 Introduction

Transmission-blocking vaccines (TBVs) target the extracellular sexual stages of the parasite that develop within the mosquito midgut or mosquito midgut ligands that interact with the parasite during its development within the mosquito. Immune responses raised by TBVs exploit the relatively large 'window of opportunity' (~24 hours) presented during parasite transition from gametocyte to gamete to ookinete[517,573]. During this transition, the parasite remains extracellular; hence numerous targets are readily accessible to antibodies or drugs, allowing interruption of parasite development. Evidence suggests that transmission-blocking activity (TBA) is predominantly antibody mediated[74,213] with host antibodies shown to persist for as long as 24 hours within the midgut at titres lethal to the parasite[209]. In order to determine whether a candidate antigen possesses TBA, functional activity is commonly assessed in mosquito feeding assays (MFAs).

The functional assays in use include mosquito feeding assays such as the standard membrane feeding assay (SMFA), direct membrane feeding assay (DMFA) and direct feeding assay (DFA). In these assays, the biological endpoint measured is a disruption of oocyst development. Aside from evaluating oocyst development, assays can also evaluate inhibition of exflagellation[321,377,386,387] or ookinete development[395,396] (ookinete development assay, IVOA). The features, merits and demerits of each assay are discussed at length in the introduction (**Chapter 1,** section **1.8.**). Owing to a greater potential for standardisation, the SMFA is currently the 'gold standard' assay[390–392]. However, the DMFA and DFA are also essential as they better replicate field conditions and hence present a more realistic view of the expected efficacy of a TBV in the population[378,379].

TBA can be presented as either the reduction in oocyst burden in the mosquito (oocyst intensity) or reduction in the number of infected mosquitoes (oocyst prevalence). Despite reporting different endpoints, these two metrics provide complementary information to assess the efficacy of a TBI. From a public health perspective,

reductions in oocyst prevalence are thought to more readily predict a reduction in the potential for transmission[405,574,575]. This is because mosquitoes with low numbers of oocysts can still develop sporozoites and transmit malaria[574]. To challenge this assertion, studies have shown that not all oocysts rupture to release sporozoites[576–579]. Hence in low-density infections, typical of field-caught mosquitoes, failure of oocyst rupture may render a mosquito non-infectious. Solely reporting reductions in oocyst prevalence may thus underestimate the TBA of an intervention. However, Stone *et al*. (2013) showed little impact of oocyst non-rupture in infections with low oocyst densities, with only 14% of the oocysts failing to rupture and nearly all ruptured oocysts resulting in salivary gland sporozoites[574]. Therefore, oocyst prevalence may not significantly overestimate infectivity.

Methodological constraints impacting the accuracy of estimates of reduction in oocyst prevalence and intensity also need to be considered when assessing TBV efficacy. The estimation of TBA depends on parasite exposure which is assessed by determining mosquito infection rates in the absence of the intervention under investigation, i.e. in control mosquitoes. The average oocyst number in control mosquitoes exerts more influence on estimates of reduction in oocyst prevalence than oocyst intensity in the SMFA. Demonstrating reductions in prevalence where the infection intensity is high (>50 average oocyst counts in control mosquitoes) even when significant reductions in oocyst intensity are observed[391,406,410] is challenging. While oocyst counts in the field are on average below 5 per mosquito[580–583], infection intensity is much higher during experimental infection and is hard to control and standardise between assays[410]. Therefore, as estimates of reduction in oocyst intensity are more robust to fluctuations in parasite exposure, oocyst intensity may be a better metric to use when comparing candidate antigens during evaluation and ranking of TBV candidates using the SMFA[405,410].

Estimates of reduction in oocyst intensity are, however, not impervious to experimental variation. The overdispersed nature of oocyst burden impacts the precision of oocyst intensity estimates[391]. Indeed, Medley *et al*. (1993) in a pooled analysis of SMFA data, showed that >70% of all oocysts were found in only10% of the mosquitoes dissected[409]. Where an intervention has high efficacy, more significant reductions in oocyst intensity result in decreased parasite aggregation, and hence more precise estimates are observed[391]. Conversely, interventions with low efficacy will

show much higher variability in efficacy estimates in replicate assays. Precision and accuracy can be increased by dissecting more mosquitoes to increase power[409] or by carrying out multiple feeds with varied experimental conditions (for instance, different sources of infected blood and parasite exposures)[391,410]. Analysis of the data can then use mixed models to take into account the effect of these experimental variables on the observed infection levels, thus providing more robust estimates[391].

As neither oocyst prevalence nor oocyst intensity is a 'perfect' metric, and both provide relevant information by which to evaluate the efficacy of a TBV candidate, it is strongly recommended that both metrics be reported[391]. Additionally, noting the impact of parasite exposure on TBA estimates, the average number of oocysts in the control group should also be reported as it provides critical information with which to interpret efficacy estimates.

6.2 Rationale

The limited number of lead vaccine candidates necessitates the identification and characterisation of novel TBV candidates. So far, Pfs25 and Pfs230 are the only antigens in clinical trials (Pfs25 ClinicalTrials.gov Identifier: NCT01867463, and Pfs230 NCT02334462 and NCT02942277. Data on the efficacy of Pfs230 from the trials are currently publicly unavailable. Results from clinical trials of Pfs25 have thus far shown limited immunogenicity with the induction of low titre short-lived responses[296,376]. A more immunogenic TBV is required, and therefore efforts to identify promising candidates to add onto the development pipeline should be intensified.

In malaria-exposed individuals, responses to gametocyte antigens other than Pfs230 and Pfs48/45 have been identified and associated with TBA strongly indicating that there are other antigens capable of mediating TBA[282]. This work seeks to evaluate a set of relatively novel candidate antigens (identified in **Chapter 4**) as potential TBV candidates. These candidates encompass antigens expressed over the developmental stages of the parasite within the mosquito midgut. In this way, the chances of identifying targets that induce antibodies (either by natural parasite exposure or by vaccination) which can disrupt the parasite during this extended window of opportunity are increased.

To increase the chances of producing recombinant protein in their native conformation, multiple protein expression platforms with previously reported success in producing properly folded protein were chosen. Sera raised against the gametocyte antigens were tested in the SMFA as a primary functional assay. Testing candidates against *P. falciparum* by DFA or DMFA requires gametocyte positive blood from human volunteers which requires extensive identification of such individuals and ethical considerations[378,379]. Therefore, candidates with proven TBA efficacy may be preferable for testing using the DFA or DMFA.

On the other hand, the gamete and ookinete antigens investigated were based on the rodent parasite, *P. berghei*, as it is challenging to produce sufficient quantities of *P. falciparum* ookinetes for *in vitro* assays. The DFA was chosen as a practical primary assay to assess TBA, with the IVOA also used as a complementary assay. An advantage of the IVOA is that it can shed light on the biological function of a candidate antigen as it primarily assesses inhibition exerted at the point of gamete to ookinete development[377]. Candidate antigens thus identified can then be further tested in SMFAs at various antibody titres and parasite exposures to ascertain TBA. The ability to triage candidates in this way would provide a practical number of candidates to screen in an SMFA where various variables are to be investigated.

6.3 Objectives

The main objective of this work was to evaluate the transmission-blocking activity of the identified sexual stage antigens (refer to **Chapter 4**).

6.3.1 Specific objectives

- Raise antibodies against the gametocyte, gamete and ookinete antigens in rodents.
- Assess the immunogenicity of antisera against the gametocyte antigens using immunoassays.
- Assess the functional activity of antibodies to the gametocyte antigens using the SMFA and of antibodies to the gamete and ookinete antigens using both the DFA and IVOA.

6.4 Materials and Methods

6.4.1 Materials

A summary of commercially available reagents is provided in **9.4 Appendix 4** and the recipes for the buffers and solutions used in this study provided in **9.5 Appendix 5**.

6.4.2 Methods

6.4.2.1 Vaccination regimen

For the functional work, the proteins prepared in **Chapter 4** were used for vaccinations, a summary of the antigens investigated is presented in **Table 6.1**. All procedures for the mouse studies were carried out following the UK Animals (Scientific Procedures) Act (UK Home Office License PPL 70/8788) and were approved by the Imperial College Animal Welfare and Ethical Review Body. This work was carried out at Imperial College over four months. Six to eight-week-old BALB/c mice were vaccinated with the antigens using a protein-in-adjuvant prime-boost regimen; a vaccine delivery platform that comprises a recombinant protein formulated in an adjuvant to enhance its immunogenicity[584] and has been widely applied in the evaluation of TBV candidates[291,318,326,489]. I aimed to vaccinate five mice per antigen, however, owing to limited quantities of some antigens and the need to obtain sufficient quantities of sera for analysis, the number of mice immunised per antigen ranged from three to ten.

The mice were anaesthetised and then vaccinated subcutaneously with the antigens two times following a three-week interval as prime and boost vaccine injection administrations respectively. Fifty μg of protein prepared in TiterMax Gold® Adjuvant (mixed at a ratio of 1:1) was vaccinated at the prime and 30 μg of protein at the boost. TiterMax Gold® is a potent water-in-oil emulsion adjuvant consisting of a block copolymer, CRL-8300, squalene and a sorbitan monooleate[585]. This adjuvant is stable, can be used with a variety of proteins, is minimally reactogenic, can stimulate complement activation and induce high antibody titres[586]. The use of TiterMax Gold® in rodent[301,587], simian[588] and avian[589] vaccinations has been shown to induce potent immune responses. Two weeks after the boost, sera were harvested from the mice by terminal anaesthesia and cardiac puncture. Sera from non-vaccinated controls were also harvested for use in the functional assays.

Table 6.1: Summary of antigens for mice vaccination

Candidate name	Gene ID	Parasite stage	Parasite species	Expression system*	Mice vaccinated (N)
Pfs230	PF3D7_0209000	Gametocyte	*P. falciparum*	WGCFS	5
CVMPPP	PF3D7_1314500	Gametocyte	*P. falciparum*	Mammalian	10
PEB-P	PF3D7_0303900	Gametocyte	*P. falciparum*	Mammalian	8
PSOP1	PF3D7_0721700	Gametocyte	*P. falciparum*	Mammalian	4
CPP4	PF3D7_0208800	Gametocyte	*P. falciparum*	WGCFS	5
MDV1	PF3D7_1216500	Gametocyte	*P. falciparum*	WGCFS	5
G377B 3D7	PF3D7_1250100	Gametocyte	*P. falciparum*	WGCFS	4
G377B PfKE04	PF3D7_1250100	Gametocyte	*P. falciparum*	WGCFS	9
PSOP25 3D7	PF3D7_0620000	Gametocyte	*P. falciparum*	WGCFS	10
PSOP25 PfKE04	PF3D7_0620001	Gametocyte	*P. falciparum*	WGCFS	6
P28	PBANKA_0514900	Gamete and ookinete	*P. berghei*	Bacterial	5
PBCPP2	PBANKA_1463900	Gamete and ookinete	*P. berghei*	Bacterial	4
PBCPP3	PBANKA_1112700	Gamete and ookinete	*P. berghei*	Bacterial	4
SOAP	PBANKA_1037800	Gamete and ookinete	*P. berghei*	Bacterial	5
PH^{+}	PBANKA_0417200	Gamete and ookinete	*P. berghei*	Bacterial	5
CHT1	PBANKA_0800500	Gamete and ookinete	*P. berghei*	Bacterial	5
THX	PBANKA_0942500	Gamete and ookinete	*P. berghei*	Bacterial	3
VAMP	PBANKA_1303700	Gamete and ookinete	*P. berghei*	Bacterial	5
SEAP	N/A	Control	N/A	Mammalian	10
GST	N/A	Control	N/A	WGCFS	3

* WGCFS – wheat germ cell-free expression system.
$^{+}$ For PH, one mouse was culled after vaccination and hence only four mice proceeded to DFA analysis

6.4.2.2 Endpoint enzyme-linked immunosorbent assay (ELISA)

Endpoint ELISAs were conducted to determine the antigen-specific vaccine-induced IgG antibody titres. Recombinant protein corresponding to each antigen under investigation was first diluted down to 2 µg/ml in PBS before coating onto an ELISA plate. The plate was then left at room temperature overnight. The next day, the wells of the plate were washed four times using PBS/Tween (PBS/T) before 100 µl of blocking buffer was added and the plate incubated at room temperature for two hours. Five hundred microlitre stocks of sera at 1:500 dilution from each vaccinated mouse, collected at the five-week post-prime time point, were then prepared. For PEB-P, it was necessary to use a higher serum dilution series of 1:1000 to 1: 2187000 for PEB-P to determine the endpoint titre owing to the high titres observed for some of the mice. Pooled sera from naïve mice were prepared similarly as a negative control. After two hours of blocking, the blocking buffer was discarded from the plate, and 300 µl of diluted sera (from the 500 µl stock) added to the wells of row A in duplicate (i.e. serum added from one mouse added to wells A1 and A2, and from another added to A3 and A4 (**Figure 6.1**). Two hundred microlitres of blocking buffer were added to all the wells of rows B – H, after which 100 µl of diluted serum was withdrawn from row A and serially diluted down the plate.

	1	2	3	4	5	6	7	8	9	10	11	12	Serum Dilution
A	Naïve 1	Naïve 1	Mouse 1	Mouse 1	Mouse 2	Mouse 2	Mouse 3	Mouse 3	Mouse 4	Mouse 4	Blank	Blank	1 in 500
B	Naïve 1	Naïve 1	Mouse 1	Mouse 1	Mouse 2	Mouse 2	Mouse 3	Mouse 3	Mouse 4	Mouse 4	Blank	Blank	1 in 1500
C	Naïve 1	Naïve 1	Mouse 1	Mouse 1	Mouse 2	Mouse 2	Mouse 3	Mouse 3	Mouse 4	Mouse 4	Blank	Blank	1 in 4500
D	Naïve 1	Naïve 1	Mouse 1	Mouse 1	Mouse 2	Mouse 2	Mouse 3	Mouse 3	Mouse 4	Mouse 4	Blank	Blank	1 in 13500
E	Naïve 1	Naïve 1	Mouse 1	Mouse 1	Mouse 2	Mouse 2	Mouse 3	Mouse 3	Mouse 4	Mouse 4	Blank	Blank	1 in 40500
F	Naïve 1	Naïve 1	Mouse 1	Mouse 1	Mouse 2	Mouse 2	Mouse 3	Mouse 3	Mouse 4	Mouse 4	Blank	Blank	1 in 121500
G	Naïve 1	Naïve 1	Mouse 1	Mouse 1	Mouse 2	Mouse 2	Mouse 3	Mouse 3	Mouse 4	Mouse 4	Blank	Blank	1 in 364500
H	Naïve 1	Naïve 1	Mouse 1	Mouse 1	Mouse 2	Mouse 2	Mouse 3	Mouse 3	Mouse 4	Mouse 4	Blank	Blank	1 in 1093500

Figure 6.1: Template used for the Endpoint ELISA. Naïve – pooled sera from non-immunised mice used as a negative control; Blank – wells where no antigen or sera were added to account for the background absorbance of the plate during the analysis of optical density values.

The plate was then incubated at room temperature for two hours before being washed four times with PBS/T. Fifty microlitres of secondary antibody (goat anti-mouse whole IgG conjugated to HRP diluted 1 in 5000 in PBS/T was then added to the plate, and the plate incubated at room temperature for one hour. The plate was then washed four times with PBS/T and 100 µl of OPD substrate added per well before incubating

the plate to allow colour development. The plate was then read three times at 10-minute intervals (10 minutes, 20 minutes and 30 minutes). This was done to identify the timepoint at which the maximal signal was attained. From the preliminary endpoint trials, 30 minutes was determined as the incubation time that allowed maximal signal detection, and was the incubation time used subsequently. The endpoint titre was then calculated as the x-axis intercept of the dilution curve at which the optical density (OD) of the wells with test sera reached that of the naïve sera plus three times the standard deviation (SD).

6.4.2.3 Standardised ELISA

Standardised ELISAs were carried out as described by Miura *et al*. (2008)[562]. This was done to allow for quantification of vaccine-induced antibody titres in arbitrary ELISA units (AUs). The day before the standardised ELISA, an ELISA plate was coated with the respective antigen for which a standard was being prepared, and incubated overnight at 4°C. The next day, the plate was washed four times with PBS/T before blocking buffer was added to the plate and the plate incubated at room temperature for two hours. Sera standards for which AUs were to be determined were prepared by pooling an equal volume of sera from three mice with the highest endpoint titres (mice vaccinated with the same antigen). Two 15 ul aliquots were prepared for use, and from these, duplicate 1:1000 dilutions were prepared. The 1:1000 dilution was then serially diluted up to a concentration of 1:512000. The blocking buffer was aspirated from the wells before 100 µl of the serially diluted sera pool was added to wells 1 – 10 of rows A and B (duplicate samples run in row B (**Figure 6.2**). The individual sera were then diluted, and 100 µl run as a sample on the rest of the plate in duplicate at dilutions of 1:2000, 1:4000 and 1:8000.

	1	2	3	4	5	6	7	8	9	10	11	12		
A	1:1000	1:2000	1:4000	1:8000	1:16000	1:32000	1:64000	1:128000	1:256000	1:512000	Blank	Blank		
B	1:1000	1:2000	1:4000	1:8000	1:16000	1:32000	1:64000	1:128000	1:256000	1:512000	Blank	Blank		
C	Mouse 1	Mouse 2	Mouse 3	Mouse 4	Mouse 5		NIH	Naïve						
D	Mouse 1	Mouse 2	Mouse 3	Mouse 4	Mouse 5		NIH	Naïve						
E	Mouse 1	Mouse 2	Mouse 3	Mouse 4	Mouse 5		NIH	Naïve						
F	Mouse 1	Mouse 2	Mouse 3	Mouse 4	Mouse 5		NIH	Naïve						1 in 2000
G	Mouse 1	Mouse 2	Mouse 3	Mouse 4	Mouse 5		NIH	Naïve						1 in 4000
H	Mouse 1	Mouse 2	Mouse 3	Mouse 4	Mouse 5		NIH	Naïve						1 in 8000

Figure 6.2: Template used for the Standardised ELISA. Naïve – pooled sera from non-vaccinated mice used as a negative control; Blank – wells where no antigen or sera were added to account for the background absorbance of the plate during the analysis of optical density values; NIH – pooled sera prepared from all mice vaccinated with a respective antigen.

Additionally, a general pool of sera prepared by combining sera from mice vaccinated with a specific antigen (regardless of titre) was also quantified alongside the individual sera. The volume of serum contributed by each mouse to this general pool varied depending on the volume of serum harvested from each mouse. The goal was to prepare a 1 ml pool of sera against each antigen for functional assays. Sera were pooled to provide a sufficient volume of purified IgG for the SMFA (see section **6.4.2.5** below). After adding the sera, the plate was incubated at room temperature for two hours after which the plate was washed, secondary antibody added (goat anti-mouse whole IgG conjugated to HRP), and the plate incubated for one hour. Signal was detected by adding 100 µl of OPD to the plate and incubating for 15 minutes before colour development was stopped by adding 25 µL of 2M H_2SO4. Absorbance was then read at 492 nM to determine the OD. An independent repeat of the standardised ELISA was carried out on a separate day using the second aliquot of the standard.

In order to compute the AUs, the starting dilution of 1:1000 was assigned 20 AUs, and a four-parameter curve fitted from data generated from the diluted standard. AUs of the standard were determined using the coefficients of the four-parameter curve (lowest asymptote, highest asymptote, point of inflection and Hill's slope). The average of the AUs from the two independent runs was calculated as the AUs of the prepared standard, provided the difference between two runs did not exceed 30%. If the difference between AUs exceeded 30%, then a second set of two independent runs was carried out. Repeat experiments were carried out (two independent replicates) for the standards prepared for G377B 3D7 and MDV1. The AUs of the individual sera and general pool were then read off the standard curve generated from the standard.

6.4.2.4 Western blots with immune sera

Western blot analysis was carried out as described in **4.4.5.2.(a)**, with a few modifications. The primary antibody used was the general pool of immune sera diluted 1:10000 in blocking buffer (TBS/T containing 4% skim milk). The membrane was first incubated with the primary antibody overnight at 4°C; on the next day, the membrane was washed three times with TBS/T and incubated with secondary antibody (goat anti-mouse whole IgG conjugated to HRP) diluted at 1:5000 for one hour. The membrane was then washed and visualised as described. This was carried

out for CVMPPP, PEB-P, SEAP and G377B 3D7 where sufficient protein quantities were available.

6.4.2.5 Standard membrane feeding assay (SMFA) – *P. falciparum* candidates

SMFAs, against *P. falciparum* candidate antigens, were carried out at the reference laboratory for this assay, Laboratory of Malaria and Vector Research, National Institutes of Health (NIH) by Dr Kazutoyo Miura. The SMFAs were carried out using a standardised protocol as described in Miura *et al*. 2013[410]. Briefly, IgG was purified from the 1 ml pool of mouse sera raised against each of the antigens (**Table 6.1**) and adjusted to a concentration of 750 μg/ml. A culture of mature stage V gametocytes was first pelleted, and the culture media replaced with normal human serum (with complement) and red blood cells and adjusted to between 0.15 – 0.2% gametocytaemia and 50% haematocrit. Sixty μl of the test sera were mixed with 200 μl of the gametocyte mixture and this mixture fed to 50 female *Anopheles stephensi* mosquitoes through a membrane feeder. In addition to the test sera, monoclonal antibody 4B7 (whose target is the TBV candidate Pfs25) that has demonstrated potent transmission-blocking activity[323] was included as an internal positive control. For the negative controls, mosquitoes were either fed with naïve mouse IgG (adjusted to the same concentration as the purified IgG from mice vaccinated with the test antigens), or with naïve normal human serum (NHS). TBA was then estimated using either the naïve mouse IgG control (for test antigens) or NHS control (for mAb 4B7). After eight days, 20 mosquitoes per test sera were dissected, and oocysts counts recorded. Assays were valid only if mosquitoes fed without 4B7 or normal mouse sera had a mean of four or more oocysts per mosquito.

6.4.2.6 Direct feeding assay – *P. berghei* candidates

In order to assess the efficacy of the sera against the *P. berghei* candidate antigens, direct feeding assays were carried out on mice previously vaccinated with the gamete and ookinete antigens on the day that sera were to be harvested (5 weeks post-vaccination). The DFAs were carried out by Dr Andrew Blagborough's team at his lab in Imperial College London. Six days prior to the assay, mice were injected intraperitoneally with 200 μl of phenylhydrazine (PH) to stimulate reticulocytosis; this has been shown to promote and increase gametocyte production[395]. Three days

before the assay, the mice were infected with *P. berghei* ANKA strain, clone 2.34. Parasitaemia was measured by microscopy in all mice prior to the mosquito feeds to ensure that all mice (control and test mice) were successfully infected. The day before the assay, 50 mosquitoes were transferred into a feeding cup, 5 cups (one for each mouse) were prepared for each test serum. On the day of the assay, the mice were anaesthetised and placed on top of a cup of mosquitoes. Gametocyte densities were also measured in all mice on the day of the feed, and the densities are provided in **9.910 Appendix 10**. Gametocyte densities varied per mouse, with no discernible patterns between cohorts of vaccinated mice and gametocytaemia. The gametocyte densities observed were typical in terms of scale and range with previous infections carried out at the Blagborough lab (Blagborough A, Personal communication). Feeding was then allowed to repletion. The cups of mosquitoes were then returned to the incubator. The next day, the mosquitoes were examined to detect unfed or partially fed mosquitoes and those that had not taken a blood meal taken out of the cups. The mosquitoes were then maintained on 8% (w/v) fructose, 0.05% (w/v) *p*- aminobenzoic acid at 19 – 22°C and 50 – 80% relative humidity for ten days before they were dissected. After this, oocysts counts per mosquito and the number of infected mosquitoes per antigen were determined.

6.4.2.7 *In vitro* ookinete conversion assay (IVOA)

The *in vitro* ookinete conversion assay was carried out as described by Blagborough *et al*. (2013)[395]. Test sera obtained from mice vaccinated with the gamete and ookinete antigens were prepared in RPMI containing 20% fetal calf serum (FCS) at a final volume of 100 µl. The diluted sera were then added to the wells of a 24-well plate. Parasitised blood (20 µl) from PH-treated mice was first resuspended in 450 µl of ookinete medium to activate the culture before adding to the test sera in the 24-well plate to give final sera dilutions of 1:5. Pre-immune sera were also included alongside the test sera as a negative control. The plate was then incubated for 24 hours at 19°C. On the next day, 100 µl of culture was pelleted by centrifugation at 500 x g for three minutes and the supernatant discarded. The pellet was washed one time with ookinete medium before resuspension in 50 µl of RPMI containing Cy3-conjugated Pbs28 mAb clone 13.1[397] diluted at 1:500. The mixture was then incubated on ice for 10 minutes, and then a volume of ten microlitres was transferred onto a glass slide and covered with a coverslip and the edges sealed with petroleum jelly. Ookinetes and

macrogametes were then enumerated under a fluorescence microscope at 10 x magnification. Three biological replicates were carried out for each test serum.

6.4.2.8 Statistical analysis

Reductions in the oocyst burden (oocyst intensity) and the prevalence of infected mosquitoes (oocyst prevalence) were estimated to assess transmission-blocking activity (TBA). This was done using generalised linear mixed models (GLMM) with a zero-inflated negative binomial structure (where appropriate) for oocyst intensity, and a binomial error structure for oocyst prevalence, as described by Churcher *et al*. (2012)[391]. The percentage reduction in oocyst intensity and oocyst prevalence was then calculated as:

$$\left[1 - \left(\frac{a}{b}\right)\right] \times 100\%$$

Where *a* is the mean oocyst intensity of the test or the infection prevalence of the test and *b* is the mean oocyst intensity of the control or the infection prevalence of the control.

Additionally, for the analysis of DFA data, where information on mosquito infectivity from each vaccinated mouse (host) was available, the effect of blood source was included in the GLMM as a random effect. A Fisher's exact test was used to estimate *p* -values where oocyst prevalence in control mosquitoes was 100%.

To calculate ookinete conversion rates, the percentage of ookinetes formed relative to the original number of macrogametes was calculated as described by Blagborough *et al*. (2013)[395]. Ookinete conversion rates were compared between samples exposed to the test antisera and those exposed to sera from naïve mice using an unpaired two-sample T-test. Additionally, inhibition of ookinete conversion was calculated using the equation below:

$$\left[1 - \left(\frac{a}{b}\right)\right] \times 100\%$$

Where *a* is the mean ookinete conversion in the presence of test sera

and *b* the mean ookinete conversion in the presence of control sera.

6.5 Results

6.5.1 Gametocyte antigens

6.5.1.1 Preliminary analysis of immunogenicity

6.5.1.1.(a) Endpoint ELISA

To determine mice with the highest antibody response to the respective antigens post-vaccination, I assessed the vaccine-induced antibody titre for each mouse five weeks post the initial vaccination, two weeks after the boost to coincide with peak boost responses. Except for two of the mice vaccinated with PSOP25 3D7, all the mice seroconverted; however, there was considerable variability in vaccine-induced responses within each group of mice (**Figure 6.3**). Notably, Pfs230-C and CPP4 induced responses appeared to be comparatively low. Data from the endpoint ELISAs informed the standardised ELISAs carried out to estimate antibody titre.

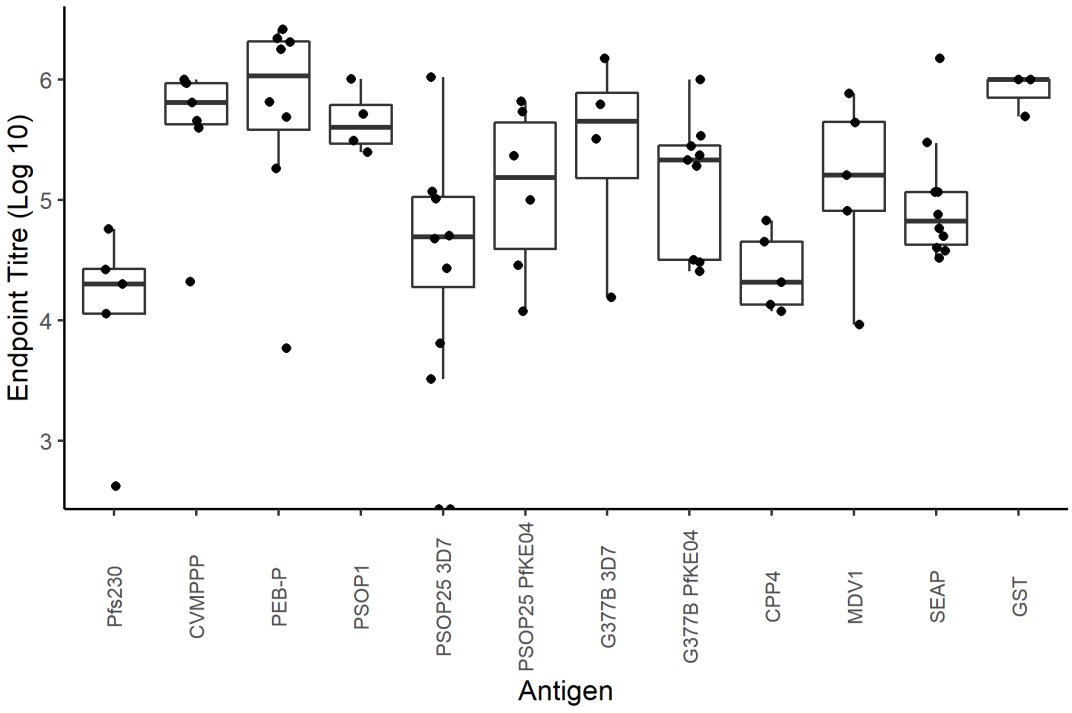


Figure 6.3: Vaccine-induced antibody responses in mice to the gametocyte antigens. Boxplots showing the log-transformed endpoint titre of vaccine-induced responses to each antigen. The boxes of boxplots display the median bound by the first and third quartiles, with the whiskers depicting the lowest and highest values (excluding outliers). Each point represents a single mouse within a group vaccinated with a single antigen.

6.5.1.1.(b) Western blot analysis

In addition to the endpoint ELISA, for a subset of the antigens, I also assessed whether vaccine-induced antibodies raised to each antigen correctly recognised its respective antigen on a western blot. I carried this out for the antigens CVMPPP, PEB-P, SEAP and G377B 3D7. From the analysis (**Figure 6.4**), each antigen tested recognised its specific antigen confirming immunogenicity. Cross-reactivity was observed for the G377 variants, where G377B 3D7 protein was recognised by sera from mice vaccinated with either G377B 3D7 or G377B PfKE04. Both G377B proteins migrate at a higher molecular weight than expected (see chapter 4.5.1.4), potentially due to the abundance of acidic amino acid residues resulting in reduced binding of sodium dodecyl sulphate (SDS) and hence they remain insufficiently denatured during electrophoresis[538–540].

Interestingly, the G377B 3D7 variant migrates at a higher molecular weight than the G377B PfKE04 despite a nine amino acid deletion in the 3D7 variant (refer to sections **4.5.1.2.(b)** and **4.5.1.4**). The reason for this remains unclear and would require further structural characterisation of the two variants. From the western blot results, it appeared that despite the variation, there could exist shared epitopes between the two variants.

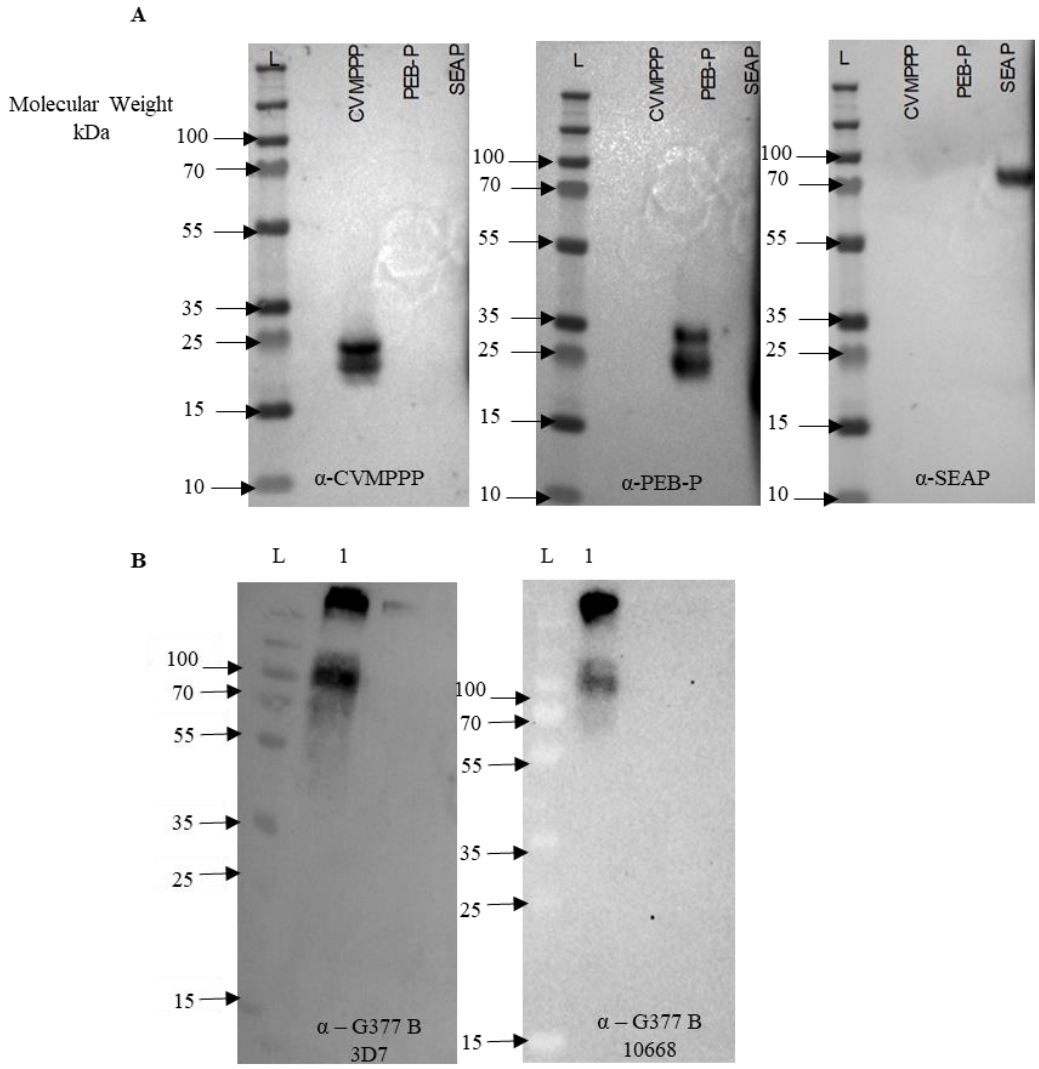


Figure 6.4: Western Blots showing the specific recognition of the recombinant antigens by immune sera. (A) Recombinant CVMPPP, PEB-P and SEAP were probed with antibodies raised in mice to each respective antigen. The blots show specific recognition of each antigen with no cross-reactivity. CVMPPP – 17.4 kDa, PEB-P – 24.4 kDa, and SEAP – 64 kDa. (B) Recombinant G377B 3D7was probed with antibodies raised in mice to both variants of the antigen. 1 – G377B 3D7 59 kDa (but migrates above 100 kDa).

6.5.1.2 Standardised ELISA

A standardised ELISA developed to accurately quantify antibody titre (reported in AUs) of a reference serum, and subsequently quantify the AUs of test sera[562] was used to quantify antibody titre in sera generated against each antigen. Based on the results of the endpoint ELISA (**Figure 6.3**), I pooled an equal volume of sera from three mice with the highest sera to create a reference serum for each antigen. I used the reference serum to estimate the AU for each serum sample from individual mice as well as a pool of sera generated from all the mice for use in functional assays. The results were similar to the endpoint analysis, with a varied immunological response seen for each antigen and mouse (**Figure 6.5**).

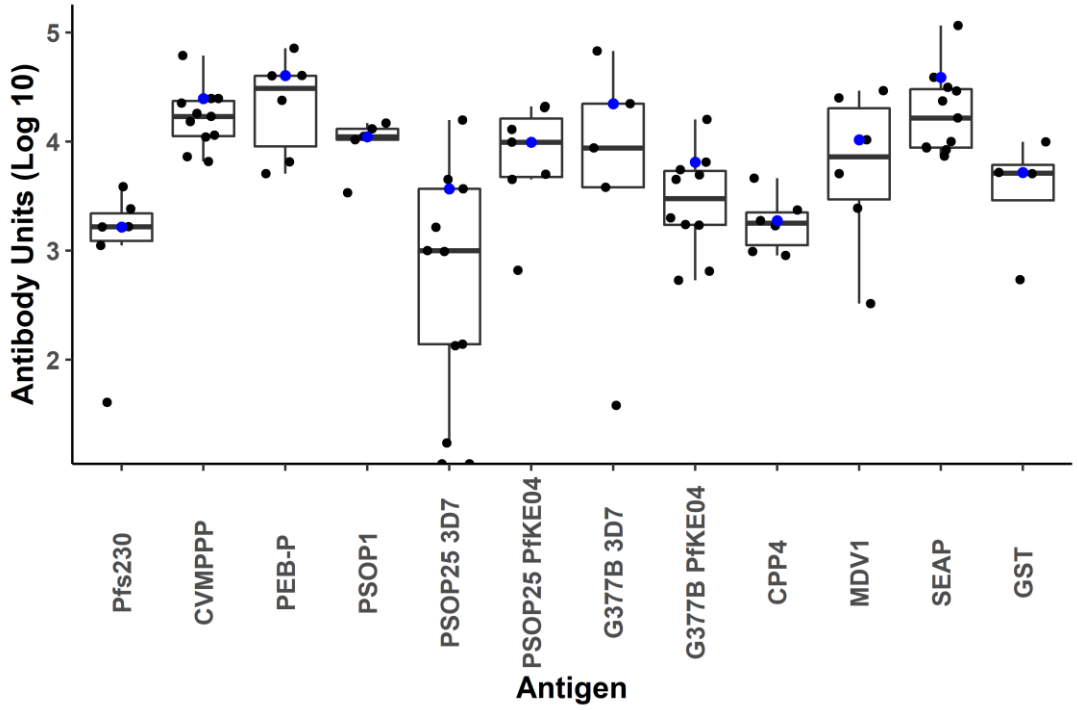


Figure 6.5: Antibody titre induced in mice post-vaccination with the gametocyte antigens. Boxplots showing the log-transformed antibody titre in arbitrary ELISA units (AUs) of vaccine-induced responses to each antigen. The AUs of the pool to NIH coloured in blue. Standardised ELISAs for three of the mice vaccinated with PEB-P were not performed as quantities of sera were limited. The boxes of boxplots display the median bound by the first and third quartiles, with the whiskers depicting the lowest and highest values (excluding outliers). Each point represents a single mouse within a group vaccinated with a single antigen.

Additionally, antibody titres were estimated for the sera pool generated for each antigen. These sera pools were for use in SMFA work described in section **6.5.1.3** below. Antibody titres varied per antigen, with PEB-P having the highest AU (40,163 AU) while Pfs230-C had the lowest AU (1,645 AU) (**Table 6.2**).

Table 6.2: Antibody of a pool of sera from mice vaccinated with the same antigen

Antigen	AU* of pooled sera
CVMPPP	24,756
PEB-P	40,163
PSOP1	11,102
SEAP	38,867
Pfs230-C	1,645
MDV1	10,379
CPP4	1,886
G377B 3D7	22,262
G377B PfKE04	6,486
PSOP25 3D7	3,689
PSOP25 PfKE04	9,877
GST	5,199

*AU – antibody units.

6.5.1.3 SMFA analysis

In order to evaluate the ability of antibodies raised against the antigens to inhibit infectivity to mosquitoes, standard membrane feeding assays were carried. *P. falciparum* (NF54)-infected gametocyte cultures were fed to mosquitoes in the presence of purified total IgG. A monoclonal antibody against Pfs25 with established transmission-blocking properties, mAb $4B7^{323}$, together with anti-Pfs230 IgG, was used as positive controls. IgG against the protein SEAP and GST were used as negative controls for proteins expressed in the mammalian and wheat germ expression systems respectively. Additionally, naïve IgG from non-vaccinated mice was used as an assay negative control from which TBA estimates were derived. Naïve normal human serum was used as a negative control for the mAb 4B7. Twenty mosquitoes were dissected to analyse oocyst counts and infection prevalence for each IgG pool. From the first SMFA, analysis of oocyst burden per mosquito indicated significant variation in the oocyst burden of mosquitoes fed on IgG against the various antigens (p <0.0001, **Figure 6.6)**.

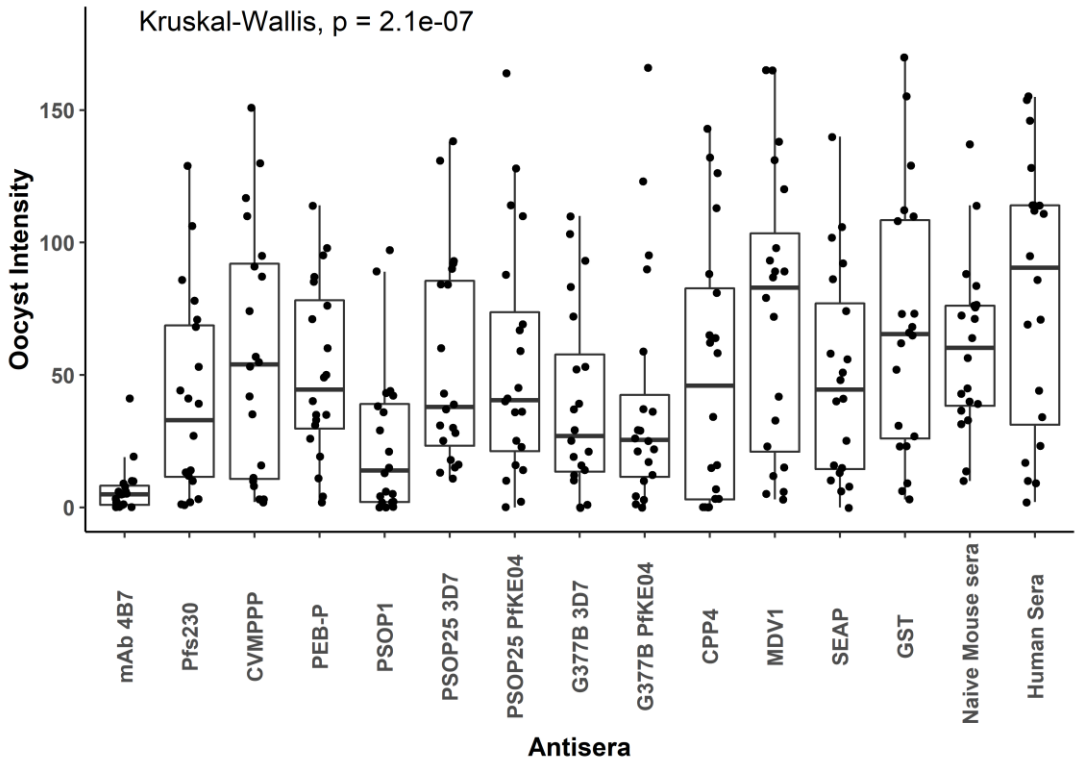


Figure 6.6: Oocyst burden in infected mosquitoes fed on IgG against the gametocyte antigens. Box plots showing the oocyst counts per mosquito group (N = 20) fed on a blood meal containing purified IgG against the gametocyte antigens mixed with *P. falciparum* NF54 gametocytes. mAb 4B7 was included as a positive control; SEAP and GST were used as negative control antigens; normal sera and human sera were used as sources of negative control IgG. The boxes of boxplots display the median bound by the first and third quartiles, with the whiskers depicting the lowest and highest values (excluding outliers). Each point represents a single mouse within a group vaccinated with a single antigen.

I then carried out analysis to evaluate the transmission-blocking activity by looking at reductions in oocyst intensity and prevalence relative to naïve sera (**Table 6.3**). I chose to use naïve mouse sera as a standard comparator as no transmission-blocking activity was observed in the mosquitoes fed on IgG from antigen controls when compared against naïve mouse IgG. Antibodies induced against PSOP1 significantly reduced oocyst intensity (59.4%, 95% CI 27.71 – 78.72, p = 0.004). Though IgG against Pfs230 (32.53% 95% CI -5.30 – 60.80%, p = 0.15), G377B 3D7 (34.36% 95% CI 0.27 – 57.31%, p = 0.12) and G377B PfKE04 (33.03% 95% CI -15.09 – 63.28%, p = 0.15) did not significantly reduce oocyst intensity, they were prioritised for a repeat SMFA to ascertain if indeed no TBA was present. No reductions in oocyst prevalence were observed for any of the IgG tested.

Mean oocyst counts in the control mosquitoes, for both the naïve mouse sera and malaria naïve human donor, were above 50 indicating high parasite exposure in the assay. Evidence of high parasite exposure can also be seen in the low estimates of reduction in oocyst prevalence. mAb 4B7 showed no reduction in oocyst prevalence despite showing a 90% reduction in oocyst intensity.

Table 6.3: Transmission-blocking activity of antisera* against the gametocyte antigens as estimated using generalised linear mixed models

Antisera	Median oocyst intensity	IQR	Inhibition of intensity	95% CI	*p* value	Infection prevalence (%)	Inhibition of prevalence	95% CI	*p* value
Pfs230	33	11.5, 68.75	32.53	-5.30, 60.80	0.15	100	0	-	.
CVMPPP	54	10.75, 92	4.33	-46.79, 42.43	0.86	100	0	-	.
PEB-P	44.5	29.75, 78.25	15.06	-23.78, 44.43	0.44	100	0	-	.
PSOP1	14	2, 39	59.4	27.71, 78.72	**0.004**	85	15	4.17, 37.50	0.2308
PSOP25 3D7	38	23.25, 85.5	10.32	-35.26, 40.05	0.59	100	0	-	-
PSOP25 PfKE04	40.5	21.25, 73.75	9.57	-36.94, 41.71	0.68	95	5	0.00, 30	1
G377B 3D7	27	13.5, 57.75	34.36	-0.27, 57.31	0.12	90	10	0.00, 32.47	0.4872
G377B PfKE04	25.5	11.5, 42.5	33.03	-15.09, 63.28	0.15	95	5	0.00, 28.18	1
CPP4	46	3, 82.75	15.97	-31.63, 53.72	0.61	80	20	5.57, 42.86	0.1060
MDV1	83	21, 103.5	-21.88	-74.59, 17.99	0.41	100	0	-	1
SEAP	44.5	14.5, 77	17.89	-27.40, 46.96	0.42	95	5	0.00, 37.50	1
GST	65.5	26, 108.5	-13.56	-64.54, 24.02	0.57	100	0	-	.
Control[1]	60.5	38.38, 76.13	-		-	100	-	-	-
mAb 4B7	5	1, 8.25	91.67	80.29, 95.59	**<0.0001**		15	4.17, 38.89	0.1154
Control[2]	90.5	31.25,114	-		-	100	-	-	-

* Total IgG purified from the antisera were used in the experiments.
[1] Control IgG from naïve mice used as a negative control for purified IgG from vaccinated mice.
[2] Control serum from a malaria naïve human donor used as a negative control for mAb 4B7.IQR – interquartile range.
(-) – transmission-blocking activity not calculated for controls; no reduction in oocyst prevalence seen.
CI – confidence interval; *p* values in bold are statistically significant at significance level 0.05 (estimated using a Fisher's exact test).

Noting the variability associated with estimates of TBA for interventions with an efficacy below 80%[391,392], a repeat SMFA was carried out using IgG against Pfs230, PSOP1 and both variants of G377B. This was done to ascertain if IgG against these antigens were indeed associated with TBA. In this experiment, anti-G377B 3D7 IgG showed reductions in oocyst intensity (60%, 95% CI 12.60 – 82.60%, $p = 0.03$ (Table 6.4)). Unfortunately, TBA observed with PSOP1 was not replicated in the second feed, and no TBA was observed with IgG against Pfs230 and G377B PfKE04. Significant reductions in oocyst prevalence were seen with IgG against G377B 3D7 (31.58% 95% CI 9.52 – 56.60%, $p = 0.04$) but not with any of the other IgG tested. In contrast to the first SMFA, parasite exposure was lower in the second SMFA (median oocyst counts in the control mosquitoes < 30, $p = 0.009$ (Wilcoxon test for difference in average oocyst density between feeds for the naïve mouse IgG control)). This was also reflected in the higher reductions in oocyst prevalence for relatively similar reductions in oocyst intensity.

Table 6.4: Transmission-blocking activity of sera against a subset of the gametocyte antigens as estimated using generalised linear mixed models

Antisera	Median oocyst intensity	IQR	Inhibition of intensity	95% CI	*p* value	Infection prevalence (%)	Inhibition of prevalence	95% CI	*p* value
Pfs230	46	22.5, 75.75	-50.28	-129.73, 5.11	0.09	100	0	-	-
PSOP1	27.5	8, 52.25	12.71	-36.83, 48.65	0.647	85	10.53	-7.69, 31.58	0.31
G377B 3D7	4	0, 15.25	60	12.60, 82.60	**0.03**	55	31.58	9.52, 56.60	**0.04**
G377B PfKE04	31	15.5, 62.25	-19.29	-100.87, 33.25	0.56	90	5.26	-9.97, 25.00	0.56
Control[1]	28	20, 42	-	-	-	95	-	-	-
mAb 4B7	0	0, 4	89.83	72.72, 96.36	**0.0002**	45	30.77	-30.62, 64.56	0.21
Control[2]	9	0, 26.05	-	-	-	65			

* Total IgG purified from the antisera were used in the experiments.
[1] Control IgG from naïve mice used as a negative control for purified IgG from vaccinated mice.
[2] Control serum from a malaria naïve human donor used as a negative control for mAb 4B7.IQR – interquartile range.
(-) – transmission-blocking activity not calculated for controls; no reduction in oocyst prevalence seen.
CI – confidence interval; *p* values in bold are statistically significant at significance level 0.05.

6.5.2 Gamete and ookinete antigens

6.5.2.1 Direct feeding assays

Similar to the gametocyte antigens, I tested whether vaccine-induced responses against the gamete and ookinete antigens possessed TBA. For this, direct feeds by mosquitoes on *P. berghei* infected mice were carried out, and oocyst intensity and prevalence estimated ten days post-feeding. Animals vaccinated against P28 were included as positive control while naïve mouse sera were used as a negative control. Oocyst burden varied significantly ($p < 0.05$) among groups of mosquitoes that fed on the different mice immunised with the antigens, as well as the control mice (**Figure 6.7**) . Notably, no correlation was seen between gametocyte densities measured before the feed and TBA outcomes (**0 Appendix 10**).

In a combined analysis of data from of all mosquitoes fed on mice vaccinated with a single antigen (**Table 6.5**), modest TBA was seen for P28 (49.12% 95% CI 34.37 – 60.55%, p <0.0001), SOAP (45.62% 95% CI 30.95 – 57.17%, p <0.0001) and PBCPP2 (36.85% 95% CI 21.29 – 49.33%, p <0.0001). Additionally, sera against P28, PH, THX, SOAP, and PBCPP2 yielded statistically significant reductions in oocyst prevalence. Based on the observed TBA, I proceeded to further analyse the functional activity of these sera in the IVOA assay, using sera from individual mice showing the highest TBA (**Table 6.6**) for the antigens P28, PH, THX, VAMP, SOAP and PBCPP2.

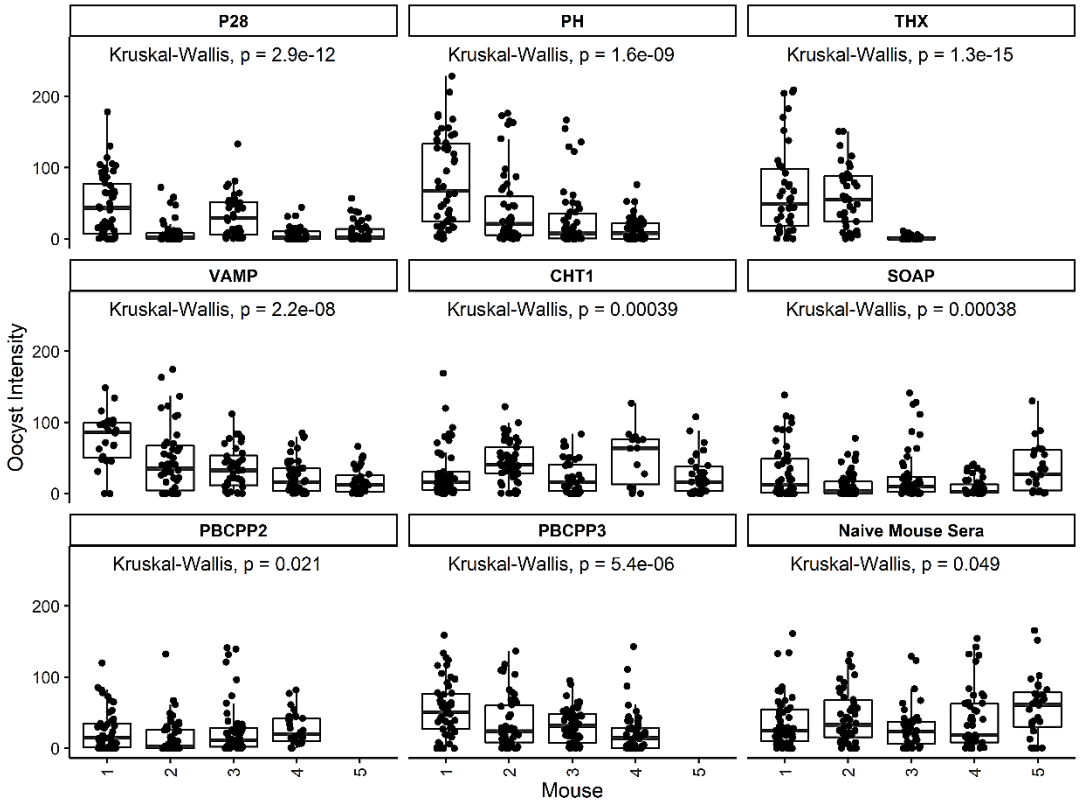


Figure 6.7: Oocyst burden in infected mosquitoes fed on sera against the gamete and ookinete antigens. Box plots showing the oocyst counts per mosquito group (N ~ 50 mosquitoes per mouse) fed on mice vaccinated with the gamete and ookinete antigens. Antisera against P28 were included as positive controls; sera from naïve mice were used as negative controls. The boxes of boxplots display the median bound by the first and third quartiles, with the whiskers depicting the lowest and highest values (excluding outliers). Each point represents a single mouse within a group vaccinated with a single antigen.

Table 6.5: Combined transmission-blocking activity of mice vaccinated with the gamete and ookinete antigens as estimated using generalised linear mixed models

Antisera	Median oocyst intensity	IQR	Inhibition of intensity (%)	95% CI (%)	*p* value	Infection prevalence (%)	Inhibition of prevalence (%)	95% CI (%)	*p* value
P28	5	0, 29	49.12	34.37, 60.55	**<0.0001**	70	21.05	6.28, 46.25	**<0.0001**
PH	18	3, 52	-2.53	-29.30, 18.70	0.833	82	8.24	0.11, 28.91	**0.04**
THX	17	1, 70.5	11.9	-20.93, 35.81	0.43	79	10.02	0.28, 38.59	**0.03**
CHT 1	24	7, 52	17.02	-0.79, 31.68	0.06	87	0.39	-3.50, 13.54	0.92
VAMP	28	4, 60	1.85	-18.89, 18.98	0.8483	84	6.1	-0.93, 22.70	0.13
SOAP	7	1, 29	45.62	30.95, 57.17	**<0.0001**	78	14.59	3.56, 34.63	**0.0007**
PBCPP2	13	1, 33	36.85	21.29, 49.33	**<0.0001**	79	12.01	1.94, 31.96	**0.006**
PPBCPP3	27	6, 55.25	1.28	-19.43, 18.40	0.8943	84	5.68	-1.07, 22.23	0.15
Control[1]	29	9, 62.25	-	-	-	88	-	-	-

[1] Control sera from naïve mice.
IQR – interquartile range.
(-) – transmission-blocking activity not calculated for controls.
CI – confidence interval; *p* values in bold are statistically significant at significance level 0.05.

Table 6.6: Transmission-blocking activity of individual mice vaccinated with the gamete and ookinete antigens as estimated using generalised linear mixed models

Antisera	Mouse*	Median Oocyst Intensity (IQR)	Inhibition of Intensity (lower, upper 95% CI (%))	*p* - value	Infection prevalence (%)	Inhibition of Prevalence (lower, upper 95% CI (%))	*p* - value
P28	1	43.5 (7.25, 77.25)	-32.41 (-87.5, 6.5)	0.1138	84	5.04 (-3.13, 29.18)	0.3924
	2	2 (0, 8)	70.3 (54.71, 80.52)	**<0.001**	60	32.17 (10.65, 60.93)	**<0.001**
	3	29 (6, 51)	11.52 (-37.95, 43.25)	0.5890	93	-4.78 (-6.94, 15.62)	0.4308
	4	1.5 (0, 10)	80.41 (70.11, 87.16)	**<0.001**	56	36.7 (13.49, 64.99)	**<0.001**
	5	2 (0, 13.75)	75.45 (58.8, 85.37)	**<0.001**	56	36.7 (13.49, 64.99)	**<0.001**
PH	1	67.5 (24, 133.5)	-99.65 (-179.3, -42.72)	**<0.001**	96	-8.52 (-7.85, 6.23)	0.1300
	2	21 (5, 59.25)	-0.13 (-44.28, 30.51)	0.9945	90	-1.74 (-5.75, 18.41)	0.7574
	3	7.5 (0.25, 35.5)	10.85 (-44.1, 44.84)	0.6394	74	16.35 (1.98, 44.25)	**0.0108**
	4	8 (0, 21.75)	56.7 (36.76, 70.35)	**<0.001**	66	25.39 (6.71, 54.3)	**<0.001**
THX	1	49 (18.25, 98)	-69.11 (-141.84, -18.25)	**0.0040**	93	-4.97 (-6.98, 14.95)	0.4074
	2	55 (24, 88)	-26.96 (-81.2, 11.05)	0.1885	98	-10.28 (-8.29, 7.09)	0.1106
	3	0 (0, 2)	94.87 (91.45, 96.93)	**<0.001**	48	46.04 (19.32, 73.19)	**<0.001**
CHT 1	1	16 (5, 30.25)	28.75 (-1.98, 50.22)	0.0639	94	-6.26 (-7.23, 10.38)	0.2597
	2	40.5 (28, 65.5)	-5.15 (-45.57, 24.05)	0.7624	92	-4 (-6.52, 14.46)	0.4727
	3	16 (3, 40.5)	23.49 (-19.24, 50.9)	0.2370	79	10.14 (-1.47, 38.73)	0.1313
	4	63.5 (12.75, 76)	-36.49 (-133.59, 20.24)	0.2564	86	3.1 (-6.09, 45.69)	0.7577
	5	16 (3, 37.5)	45.99 (10.46, 67.42)	**0.0169**	84	4.62 (-4.09, 33.56)	0.5109
VAMP	1	86 (50.5, 99)	100 (-inf, 100)	0.9999	93	-4.67 (-7.27, 22.59)	0.5236
	2	35 (4, 67)	-26.89 (-82.28, 11.67)	0.1975	78	12.08 (-0.29, 39.85)	0.0615
	3	32.5 (11.5, 53.25)	-5.58 (-63.42, 31.78)	0.8074	89	-0.48 (-5.71, 24.46)	0.9409
	4	16 (3, 35)	37.75 (11.37, 56.28)	**0.0085**	82	7.31 (-2.18, 32.46)	0.2232
	5	12 (2.5, 25.5)	61.34 (35.69, 76.76)	**<0.001**	77	12.48 (-1.03, 44.11)	0.0945

Antisera	Mouse*	Median Oocyst Intensity (IQR)	Inhibition of Intensity (lower, upper 95% CI (%))	*p* - value	Infection prevalence (%)	Inhibition of Prevalence (lower, upper 95% CI (%))	*p* - value
SOAP	1	12.5 (1.25, 48.5)	8.78 (-32.79, 37.34)	0.6313	76	14.09 (0.89, 41.49)	**0.0256**
	2	3 (0, 16.5)	61.89 (42.6, 74.7)	**<0.001**	62	29.91 (9.3, 58.8)	**<0.001**
	3	10 (2.25, 23.5)	21.71 (-24.62, 50.81)	0.3022	78	11.83 (-0.17, 38.6)	0.0566
	4	2.5 (1, 12.75)	76.03 (64.92, 83.62)	**<0.001**	76	14.09 (0.89, 41.48)	**0.0255**
	5	27 (4, 61)	30.67 (-17.89, 59.23)	0.1763	96	-8.52 (-8.14, 19.64)	0.2741
PBCPP2	1	15 (1, 34.5)	27.55 (-4.54, 49.78)	0.0849	78	11.83 (-0.17, 38.6)	0.0566
	2	3 (0, 25.75)	53.59 (30.77, 68.88)	**<0.001**	68	23.13 (5.47, 51.93)	**<0.001**
	3	11.5 (2, 28.75)	18.81 (-29.4, 49.06)	0.3809	78	11.83 (-0.17, 38.6)	0.0566
	4	20 (10.25, 41.5)	34.96 (3.89, 55.98)	**0.0308**	93	-5.51 (-7.4, 19.39)	0.4304
PBCPP3	1	50.5 (27.25, 76.75)	-43.23 (-98.05, -3.59)	**0.0297**	94	-4 (-6.52, 14.47)	0.4728
	2	24 (8, 60.5)	8.16 (-30.34, 35.28)	0.6339	84	5.04 (-3.13, 29.19)	0.3923
	3	31.5 (7.25, 48.25)	4.48 (-43.18, 36.27)	0.8244	88	0.52 (-4.92, 22.17)	0.9275
	4	14.5 (0, 28.75)	34.46 (4.68, 54.94)	**0.0271**	70	20.87 (4.27, 49.47)	**0.0016**
Control[1]	All	29 (9, 62.25)	-	-	88	-	-

[1] Control sera from naïve mice.
IQR – interquartile range.
(-) – transmission-blocking activity not calculated for controls.
CI – confidence interval; *p* values in bold are statistically significant at significance level 0.05.
* Mice in bold had significant TBA above 50% and were prioritised for the IVOA.

6.5.2.2 *In vitro* ookinete conversion assay (IVOA)

For P28, PH, THX, VAMP, SOAP and PBCPP2, where individual antisera showed >50% reduction in oocyst intensity, I also evaluated TBA using the IVOA. The IVOA assesses the ability of anti-sexual stage interventions to inhibit the transition from gametes to ookinetes, thereby interrupting transmission[395,590]. As with the DFA analysis (**6.5.2.1**), P28 and SOAP antisera from individual mice also showed reductions in ookinete conversion. For P28, mean conversion rates were 50% (SD 5.36, $p = 0.01$), 33% (SD 12.63 $p = 0.02$), and 46% (SD 2.73, $p < 0.0001$) for sera from mice 2, 4 and 5 respectively (**Table 6.7)**. These conversion rates were similar to those observed for SOAP antisera (mouse 1: 47%, SD 2.91, $p < 0.0001$ and mouse 2: 43.62%, SD 2.73, $p < 0.0001$). On the other hand, sera against PBCPP2, PH, THX and VAMP though promising in the DFA analysis did not significantly reduce ookinete conversion rates.

Table 6.7: Transmission-blocking activity of individual mouse sera against a subset of the gamete and ookinete antigens (IVOA)

Antisera	Mouse	Mean ookinete conversion rate[i]	Standard deviation	*p* value	Inhibition of ookinete conversion (%)
P28	2	50.01	5.36	**0.0061**	35.88
	4	33.21	12.63	**0.0233**	57.42
	5	45.58	2.73	**<0.0001**	41.56
PH	4	80.46	6.61	0.5896	-3.17
THX	3	71.97	7.56	0.2995	7.72
VAMP	5	69.62	6.37	0.1402	10.73
SOAP	2	47.01	2.91	**<0.0001**	39.73
	4	43.62	2.73	**<0.0001**	44.07
PBCPP2	2	41.92	21.49	0.0995	46.25
Control[1]	1	78.57	5.99	-	-
	2	76.74	1.85	-	-
	3	78.66	1.84	-	-

[i] Inhibition of conversion calculated relative to conversion rates seen with naïve mouse sera. Mean of three biological replicates.
[1] Control sera from naïve mice.
CI – confidence interval; *p* values in bold are statistically significant at significance level 0.05.

6.6 Discussion

This chapter describes the work carried out to assess the functional activity of antibodies raised against the identified sexual stage antigens (refer to **Chapter 4**, sections **4.5.1.1**and **4.5.1.2**). Recombinant proteins corresponding to the candidate antigens, comprising antigens highly expressed in gametocytes, gametes and ookinetes, were produced in various expression systems and used to vaccinate mice for antibody production. The generated antibodies were then used in functional assays to evaluate their potential of the identified antigens as TBV candidates. To this end, I carried out SMFA analysis using antisera against the gametocyte antigens and carried out DFA analysis using antisera against the gamete and ookinete antigens. For the DFAs, feeds were carried out on individual mice allowing assessment of TBA in individual mice as well as a pooled analysis.

Endpoint ELISAs carried out on sera raised against the gametocyte antigens revealed that the proteins were all immunogenic, with antibody responses detectable to each of the antigens. The immunogenicity was also confirmed on a subset of antigens by western blot analysis using the corresponding recombinant protein. Specific recognition of the respective antigen was observed for CVMPPP, PEB-P and G377B 3D7. Moreover, there appeared to be cross-recognition of the G377B 3D7 variant by sera from mice vaccinated with the PfKE04 variant. Sequence variation was identified between the two variants (refer to **Chapter 4** sections **4.5.1.1**and **4.5.1.4**) which could impact protein structure leading to the induction of variant-specific responses. This can be detrimental for vaccine efficacy in the field, as has been seen for highly polymorphic asexual stage antigens such as AMA1 and MSP-2[254,256]. Reassuringly, the western blot analysis provided preliminary, though indirect, evidence of shared epitopes between the G377B 3D7 and G377B PfKE04 variants. Such conserved epitopes would be highly beneficial to overcoming antigenic diversity[204].

Notably, for a majority of the antigens, there was considerable variation in the antibody titre per mouse within groups of mice vaccinated with the same antigen. Variation in antibody production in mice has been described, even for inbred strains of mice such as the BALB/c used for this work[591], and could relate to variations in stochastic or environmental influences, or be driven by epigenetic or genetic factors[592]. Moreover, antibody titres induced by the different gametocyte antigens

varied, with the highest titres observed in PEB-P and CVMPPP-vaccinated mice, while the lowest titres were observed in Pfs230-C and CPP4 vaccinated mice. Structural analysis to ascertain protein conformation was not carried out for the antigens. Therefore, it is not possible to compare immunogenicity between the antigens used for this study.

For future work, vaccination protocols using optimised antigen concentrations, different adjuvants, and longer intervals between the priming dose and booster dose could be explored to increase antibody titres[593,594]. Additionally, other vaccine delivery platforms such as viral vectors that have been successfully used to induce potent functional immune responses to sexual stage antigens[289,595] could be explored. A better approach may be to combine a viral vector prime with a protein-in-adjuvant boost as this approach has been described to enhance antibody and cellular responses as well as improve the quality of antibody response (IgG isotype and avidity)[584,596,597].

The low antibody titres observed for Pfs230-C were unexpected. A previous study by Miura *et al*. (2013) reported high immunogenicity following vaccination of mice with wheat germ cell-free system-produced Pfs230-C[221]. Differences in vaccination protocol (adjuvant, mouse strain and the time interval between prime and boost) or possible inefficiencies during protein production may explain the low anti-Pfs230 titre observed in this study. While Titermax Gold® (used in this study) has been described to induce potent antibody responses[301,587–589], it is possible that when formulated with Montanide ISA 720 (Miura *et al*. study[221]) Pfs230 induces a more robust antibody response.

BALB/c mice (used in this study) have been successfully used to raise functional antibodies to a variety of *P. falciparum* antigens[291,318,386,517], including Pfs230[289]. Therefore, it is unlikely that the choice of mouse strain had a severe impact on immunogenicity in this study. Further to improving the vaccination regimen, it would also be critical to verify that the recombinant protein used in this study was produced in proper conformation. Pfs230 has a complex tertiary structure[486,487] hence improperly folded protein can impair antigen processing and presentation or lead to premature protein denaturation before encountering immune cells thereby reducing immunogenicity (reviewed in Saylor *et al*. (2020)[598] and Scheiblhofer *et al*. (2017)[599]).

The low anti-Pfs230 titres could also explain the lack of TBA observed in the first and second feeds. Pfs230 is a leading vaccine candidate with anti-Pfs230 antibodies demonstrating greater than 90% efficacy in reducing both oocyst intensity and prevalence in animal studies[213,221,289,489]. Antibody titre has been described to be a key determinant of TBA, where higher TBA is associated with higher antibody titres[213,296,322,600].

Likewise, high avidity[213,601] and IgG isotype[213] (a high IgG2/IgG1 ratio) have also been associated with higher TBA. The mosquito midgut is a ' harsh, highly proteolytic environment' hence high antibody titres and high avidity are required to efficiently inhibit parasite development[517]. Furthermore, the activity of antibodies against Pfs230 is enhanced in the present of complement[206] and therefore, as mouse IgG2 and IgG3 fix human complement better than IgG1[213,602], a high IgG2/IgG1 is thought to enhance TBA. Currently, 'gold-standard' cut-offs of titre, avidity and IgG2/IgG1 ratio above which high and reproducible TBA is achievable following vaccination with Pfs230 have not been defined.

From the results of the first SMFA analysis, only PSOP1 showed modest reduction in oocyst intensity (59%). None of the antisera against the other candidate antigens was associated with reductions in oocyst prevalence. None of these candidate antigens has been evaluated as TBV candidates in a *P. falciparum* model of infection, with only *P. berghei* PSOP25 (PbPSOP25) evaluated as a TBV candidate. Considerable TBA (> 60% and 25 – 31% reductions in oocyst intensity and prevalence respectively) was observed by Zheng *et al.* (2017) with anti-PbPSOP25 sera[302,517]. Furthermore, their work also demonstrated a role for PSOP25 protein in the maturation of ookinetes.

Unfortunately, the PSOP25 variants produced for this study showed negligible TBA, possibly illustrating a need to improve antibody titres and also ascertain that native epitopes were recapitulated in the proteins used for vaccination. Alternatively, it might be that while the rodent PSOP25 protein plays an essential role in fertilisation, the *P. falciparum* homolog may have a non-essential function. This phenomenon has been observed with Pfs47, where Pfs47 plays an essential role in fertilisation in *P. berghei* but not in *P. falciparum*[286,603].

SMFA analysis was repeated for antisera against both variants of G377B as well as PSOP1 where a trend towards modest TBA was observed in the first SMFA analysis.

In the repeat, only, G377B 3D7 showed a 60% reduction in oocyst intensity and a 32% reduction in oocyst prevalence. G377 protein is associated with the osmiophilic bodies of gametocytes[524,604], and from knock-out studies, it is believed to be vital in aiding female gametocytes egress from the erythrocyte[605] and may also be involved in oocyst formation[606]. The modest TBA observed in the second feed, and evidence for a role in sexual stage development indicate that G377B may have potential as a TBV target. However, conflicting results in the replicate SMFAs make it challenging to conclusively judge G377B and PSOP1 as a TBV candidates. Gene knockout studies of PSOP1 have not conclusively described a sexual stage phenotype, though some evidence suggests functional redundancy as the PSOP1 knockout did result in comparable oocyst and sporozoite burden as the wildtype[519,520]. Further experiments will be required to ascertain the TBA of G377B and PSOP1.

CVMPPP has been implicated as a potential TBV candidate, albeit indirectly, by Stone *et al*. (2018) who found that sera from individuals with high responses to CVMPPP were also more likely to exhibit >90% TBA[74].

Relatively high antibody titres were observed in mice vaccinated with CVMPPP and PEB-P, and therefore the lack of TBA may not be linked to antibody titre. Further analysis to confirm protein structure, surface localisation and antibody quality may provide insight into the lack of functional activity. Additionally, as both antigens were produced in the mammalian system, internal N-glycosylation sites were modified to improve protein expression[485] as N-linked glycans are thought to be absent in *P. falciparum*[512,513]. Kapulu *et al*. (2015), however, showed with Pfs48/45 that modification of N-glycosylation sites could adversely impact TBA,[289] possibly by inadvertently modifying key epitopes[607,608]. Hence this could also explain why despite high antibody titres, TBA was not observed with either CVMPPP or PEB-P.

As with G377B, MDV1 localises to the osmiophilic bodies[609] and likely has an essential role in gametocyte egress and ookinete development based on rodent malaria knock-out studies[522,523]. Antisera against MDV1 did not exhibit any TBA in this study despite relatively high antibody titres post-vaccination. Further analysis to confirm protein structure and surface localisation is warranted. Moreover, investigations into the quality of response regarding avidity and IgG isotype could indicate whether

optimising the vaccination regimen (use of a different platform or adjuvant) may enhance functional activity.

CPP4 has not been evaluated as a TBV candidate or for a role in sexual stage development. For anti-CPP4 sera, no TBA (reductions in oocyst intensity or prevalence) was observed. As with Pfs230, CPP4 was poorly immunogenic; hence the low antibody titres could explain the low TBA observed. CPP4 has been deemed essential to parasite survival owing to its refractoriness to mutagenesis[610] and therefore may warrant further investigation as a TBV candidate following optimisation of immunogenicity.

In addition to factors such as suboptimal antibody titres or improperly folded protein, the variability of the SMFA may also pose a challenge to detecting TBA. In experiments with high infection rates, high parasite exposure negatively correlates with TBA[405]. Interventions with high TBA may still be able to block transmission at high parasite exposure, but moderate TBA would be challenging to detect. In the first SMFA experiment, the mean oocyst counts in the control experiments were above 50 oocysts, which could explain the low reductions in oocyst prevalence observed. Furthermore, the limited dynamic range of the SMFA 80 – 100% complicates the ascertainment of moderate TBA[221]. The significant measurement error of the assay makes it near impossible to obtain consistent TBA estimates in repeat assays where the inhibitory activity is low[221,405]. A clear demonstration is seen in the lack of reproducibility in replicate SMFA results for Pfs230, PSOP1 and G377B antisera. A way around this is to carry out multiple feeds with different concentrations of antisera, at different gametocyte exposures and multiple blood sources[391,405,410]. However, this was not possible in this current work owing to time constraints.

To evaluate the potential of the identified gamete and ookinete antigens as TBV candidates, antisera against *P. berghei* P28 protein, homologous to Pfs25, was used as a positive control. Anti-Pb28 sera have shown greater than 90% TBA in DFA analysis[194,611,612]. From the pooled analysis, anti-Pb28 sera showed moderate reductions in oocyst intensity (49%) and prevalence (21%), however, three mice showed higher TBA (>70% and >30% reductions in oocyst intensity and prevalence respectively) in the individual analysis. The lower activity seen in comparison to other studies and with two of the mice may be linked to antibody titre, which as previously

mentioned positively correlates with TBA. Due to time constraints, I was unable to quantify antibody titres as I did with the gametocyte antigens and therefore could not assess the impact of antibody titre on the TBA observed. Another explanation could be that the protein expression platform used may have yielded a protein of lower immunogenicity. Matsuoka *et al*. (1994) observed low immunogenicity of *E. coli* produced Pb28 that resulted in 33% TBA which they hypothesised was due to inefficiently folded protein[613].

Nevertheless, there was detectable TBA in the DFA analysis with the Pb28 protein used, and this was confirmed in the IVOA analysis where anti-Pb28 sera showed between 33% - 50% reduction in ookinete conversion rates. Similar to anti-Pb28, sera against SOAP and a previously uncharacterised antigen, PBCPP2, also showed TBA in the DFA (both antigens) and IVOA (SOAP) analyses. SOAP is a secreted protein associated with ookinete micronemes that may play a role in ookinete invasion of the midgut[614]. Individual antisera against SOAP (from mice with the highest TBA) were associated with reductions of >60% and between 14 - 30% in oocyst intensity and prevalence respectively, as well as a 40% reduction in ookinete conversion rates. These results are promising and indicate that SOAP warrants further investigation as a TBV candidate. One other study that evaluated the TB potential of SOAP did not find an association with TBA[505]; this discrepancy could relate to differences in protein constructs used and their ability to recreate native epitopes. The function of PBCPP2 protein is currently unknown, and therefore an explanation for its role in mediating TBA remains unclear. However, based on data from PlasmoDB, PBCPP2 is described as a putative HSP20-like chaperone and may contain domains associated with binding heat shock proteins. Therefore, PBCPP2 may play a role in gamete development triggered under stress-conditions within the mosquito midgut[615].

There was also evidence to suggest modest TBA associated with antisera to PH, VAMP and THX (when TBA of individual mice was analysed) in the DFA but not the IVOA. Assay endpoints differ between the DFA and IVOA and hence the mechanisms by which antisera to the three antigens mediate TBA may only be evident at the ookinete-oocyst transition. PH has been evaluated as a TBV candidate by Kou *et al*. (2016). The authors observed a dose-dependent inhibition of ookinete conversion, that was almost completely blocked at the highest concentration examined, as well as approximately 50% and 10% reductions in oocyst intensity and

prevalence. Therefore, the recombinant PH protein used in this study may not have been as immunogenic, although this requires further investigation of antibody titre generated following vaccination. VAMP and THX proteins have not been investigated as TBV targets, neither has a sexual-stage phenotype been investigated by gene knock-out studies hence it is difficult to interpret the TBA seen in the context of what is known. On the other hand, though CHT1, a chitinase that aids ookinete passage through the peritrophic membrane[325], has been described to induce TBA[326] this was not reproduced in this study. Similarly, antisera to PBCPP3, an as-yet-uncharacterised protein, also did not demonstrate TBA in either of the TB assays utilised.

These preliminary analyses on the candidate gametocyte, gamete and ookinete antigens while highlighting potential TBV candidates for further investigation, also indicate a need to carry out additional analyses to (1) confirm protein conformation and immunogenicity, (2) confirm localisation to ascertain that the targets are accessible to antibodies, and (3) evaluate and optimise antibody titres induced by vaccination. Though only modest TBA was observed where TBA was detected, there is evidence to suggest that TBIs with low efficacy (below 80%) can be efficacious in the field when parasite transmission is low[401], particularly if combined with a pre-erythrocytic vaccine[313]. Moreover, unlike in experimental conditions, oocyst distribution in field-caught mosquitoes is highly overdispersed with the majority of mosquitoes harbouring fewer than five oocysts and only a few mosquitoes with a high oocyst burden[405,580–583]. Therefore, even TBVs with modest TBA can potentially have an impact in reducing parasite transmission in the field.

6.6.1 Limitations

Though the work presented here is preliminary, there is evidence to suggest the identification of a few novel TBV candidates. The TBA observed, however, was modest, with none of the candidate antigens showing above 80% TBA. Limited quantities of sera were available, and hence it was not possible to replicate the functional assays using different antibody concentrations and levels of parasite exposure, particularly for the SMFA. Such measures have been proposed as a way to reduce the uncertainty of SMFA-based TBA estimates[391,405,410].

While one might be tempted to doubt the potential of the antigens as TBV candidates, there are several other reasons for the low efficacy observed including reduced

immunogenicity arising from either failure to produce correctly folded recombinant protein efficiently or from a sub-optimal vaccination protocol. Owing to time considerations and challenges with obtaining sufficient quantities of proteins (particularly for the wheat germ produced proteins), I was unable to carry out structural analysis to verify the conformation of the proteins. Where proteins adopt complex tertiary structures (for instance Pfs230[486,487]), recreating conformational epitopes in recombinant protein is key to inducing functional antibodies[613]. Therefore, these results are preliminary and would require verification once information on the conformation of the recombinant protein is available. Structural analysis would also enable clarification of the discrepant results for Pfs230 where the recombinant protein produced appeared a target of natural immunity in malaria exposed individuals (**Chapter 5**) but did not induce high TBA.

Additionally, I chose to use a tried-and-tested protocol for vaccination; however, the results indicate that the protocol may require optimisation possibly by using a different vaccination platform, adjuvant, longer interval between prime and boost or a higher or lower antigen concentration[593,594]. Again, due to limited time, I could only measure antibody titre for the gametocyte antigens. However, as it is well described that titre is key to TBA[213,322,600], improved titres could also improve the TBA seen for the gamete and ookinete antigens. I was also unable to carry out surface localisation experiments for the candidate antigens, which could be achieved using immunofluorescence assays. Confirming the localisation of the antigens would have also aided the interpretation of the results from the functional assays, as one could then speculate whether the target antigens are indeed accessible to the TB antibodies.

6.6.2 Summary of overall findings

To summarise, the work presented here indicates that the proteins SOAP and PBCPP2 can induce TBA. For the antigens highlighted, the TBA observed (both reductions in oocyst intensity and oocyst prevalence) was modest. As these analyses are preliminary, there is potential to improve antibody titres induced during vaccination which could, in turn, improve the TBA induced by these antigens. Additionally, for G377, though not conclusive, there is early evidence to suggest that the sequence variation between 3D7 and the field isolate PfKE04 does not appear to impact immunogenicity. Though cross-recognition indicates shared epitopes that may

overcome parasite diversity in the field, G377B PfKE04 was not associated with TBA as G377B 3D7 was in the replicate SMFA. Antibody titres (**Table 6.2)** were much higher for the G377B 3D7 variant (22,000 AU) in comparison to G377B PfKE04 variant (6,486 AU), and this could have impacted the level of TBA seen with the two variants. Alternatively, the sequence variation may indeed impact TBA; further investigation is therefore required to understand the impact of the observed polymorphism.

Future work to verify protein structure can also highlight any shortcomings in recombinant protein production that could be alleviated by the use of a different protein expression platform. Furthermore, the proteins have peak expression at different stages of sexual development (gametocyte/gamete to ookinete), which can be confirmed by surface localisation and further functional assays. Therefore, there is a possibility to explore the synergy between the antigens and to identify combinations with superior TBA. The number of candidate antigens to add to the TBV testing pipeline can thus be increased.

Chapter 7

Concluding Remarks and Future Plans

7.1 Summary of main findings

The need for novel tools to add to the existing options of malaria control interventions has reinvigorated efforts to develop transmission-blocking vaccines (TBVs). To this end, three lead candidate antigens are under various stages of clinical development. Prioritisation of these candidates was not based on comparative assessments of efficacy but rather on the historical identification. Therefore, it calls in to question whether there are other antigens with equal or superior transmission-blocking activity (TBA) that should be included in the development pipeline.

Moreover, our understanding of naturally acquired transmission-blocking immunity (NA-TBI) remains incomplete. NA-TBI can provide a means to guide antigen discovery and provide useful information that could aid the eventual implementation and assessment of TBVs[203] and improve our understanding of the human infectious reservoir[74,200]. Unfortunately, the few studies looking into NA-TBI of sexual stages are not enough to provide a clear consensus on the critical features of anti-gametocyte immunity. The body of work presented in this thesis aims to address the above knowledge gaps by characterising a set of novel sexual stage antigens, spanning the gametocyte, gamete and ookinete stages, as targets of TBI.

To improve our understanding of anti-gametocyte immunity, I (1) carried out a systematic review and meta-analysis of studies that assessed responses to Pfs230 and Pfs48/45 in African populations (**Chapter 2**), (2) investigated gametocyte carriage in a longitudinally monitored cohort of children (**Chapter 3**); (3) produced and profiled immune responses to a set of newly identified gametocyte antigens as well as to Pfs230 (**Chapter 4** and **Chapter 5**). Key findings from these analyses were that:

(a) Host age is an essential factor influencing the prevalence and magnitude of antibody responses to sexual stage antigens. Evidence of a gradual increase in responses with age was most marked for Pfs230 and for G377 (domain B) but not for PEB-P indicating that only a subset of gametocyte antigens may generate stable, long-lived responses. For PEB-P, a stronger association was observed with patent gametocytaemia than with age.

(b) Concurrent parasitaemia was also an essential determinant of natural immunity to gametocyte antigens. Higher density infections boosted responses to the gametocyte antigens, though submicroscopic gametocytaemia was also seen to boost responses to Pfs230.

These results can have two important implications. Firstly, the availability of proteomic datasets and bioinformatic tools to mine them coupled with high throughput protein expression and antibody screening tools could increase the number of antigens identified with a potential role in TBI. Screening for antigens that are associated with more stable antigen responses may provide a rational criterion for selecting candidates for further evaluation of functional activity. Secondly, though these results are preliminary, a subset of gametocyte-specific antigens that are possibly associated with concurrent gametocytaemia was identified. Structural analysis to confirm the structure of the recombinant protein used for the immunoassays will be required. However, if confirmed, the identified antigens could be exploited to develop screening tools to identify individuals or populations at a higher risk for transmission[616,617]. Moreover, such screening tools could prove useful in serological assays aimed at monitoring the efficacy of TB interventions in the field. Additional information on age, transmission intensity and previous malaria exposure, variables that were associated with gametocyte carriage in the epidemiological analysis, could also be used in conjunction with the serological tools.

Another valuable insight from the epidemiological analysis was that the adoption of ACTs as first-line treatment for malaria in the Kilifi Malaria Longitudinal Rolling Cohort might have contributed to reduced post-treatment gametocyte carriage. These results are preliminary as the analysis does not provide definitive proof for a role for ACTs; however, they provide an interesting study for future investigations of the epidemiology of gametocyte carriage in the KMLRC. Previous studies have shown that ACTs can impact post-treatment gametocyte carriage and onward infectiousness to mosquitoes[462–464]. Therefore, the use of drug combinations that promote gametocyte clearance as well as the development of novel antimalarials with action against both asexual and sexual parasite stages can accelerate malaria elimination efforts.

In addition to exploring the associations between age and parasitaemia with gametocyte carriage and anti-gametocyte immunity, I also evaluated the potential for boosting of anti-gametocyte immunity following the malaria transmission season. Unfortunately, results from the seroepidemiological analyses carried out did not provide conclusive evidence of boosting of responses and could not indicate the longevity of responses to the panel of antigens tested. However, challenges in teasing out these associations highlight critical considerations for future seroepidemiological studies. First, the investigation of seasonal variation in anti-gametocyte antibody responses should possibly be done in areas where a clear demarcation exists between malaria transmission seasons. Second, the assessment of NA-TBI should ideally be done using longitudinal prospective cohort studies across all age groups where clinical, parasitological, infectivity and antibody data are assessed frequently.

Further to assessing NA-TBI, I evaluated the TBA of antibodies raised against a set of chiefly uncharacterised gametocyte, gamete and ookinete antigens (**Chapter 6**). For the gametocyte antigens, I included two antigens (G377 and PSOP25) that exhibited sequence variation between the widely studied lab isolate 3D7 and PfKE04 (a local Kilifi field isolate).

Modest TBA was observed for Pfs230, G377B (both PfKE04 and 3D7 variants) and PSOP1. Due to challenges in ascertaining moderate TBA using the SMFA, the results were only replicated for the G377B 3D7 variant. The lack of reproducibility also made it difficult to ascertain the impact of the sequence polymorphism on TBA. In one feeding experiment, both G377B variants showed comparable TBA but not in the second experiment. Encouragingly, in the immunoprofiling analysis, G377B PfKE04 and G377B 3D7 showed similar patterns of association signalling the existence of conserved epitopes that may overcome sequence diversity. However, this will require further investigation.

Previous associations between antibodies to Pfs230 and high-level transmission-blocking activity (TBA) suggest that reduced protein immunogenicity or sub-optimal vaccine delivery could explain the modest TBA observed. This was also indicated in the low antibody titres generated post-vaccination and provides a means to improve the TBA of these candidates. Encouragingly, antisera against the gamete and ookinete antigens *P. berghei* SOAP and a conserved uncharacterised antigen, PBCPP2,

exhibited TBA similar to that observed using antisera against Pb28 in both DFA and IVOA. Further investigation of their potential as TBV candidates is warranted. Some evidence of TBA was also observed with antisera against PH, VAMP and THX in the DFA. As noted previously, confirmation of the structure of the recombinant protein used to raise the antibodies for functional assays will be paramount. This will allow verification of TBA (or lack thereof) seen with the candidate antigens and allow prioritisation of candidates for optimisation of protein production, and further functional evaluation.

7.2 Recommendations for future work

The work presented in this thesis can be extended in future studies by refining the approach used to assess NA-TBI and validating the potential candidate antigens identified in this work.

7.2.1 Characterisation of NA-TBI

Despite the analysis carried out in chapters three and five, pertinent questions regarding boosting of naturally acquired sexual stage responses during the transmission season and the longevity of such responses remain unanswered. Natural boosting of vaccine-induced responses in the field is thought to be an advantage of TBV vaccines based on pre-fertilisation antigens such as Pfs230 and Pfs48/45. A TBV should remain efficacious over at least one transmission season[289,618]. However, the rapidly waning titres observed with the Pfs25-based TBV[197,296] indicates a potential challenge in achieving long-lasting protection in the field. Preliminary evidence of boosting following natural infection was shown in a study by Ouedraogo *et al*. (2018) where anti-Pfs230 and anti-Pfs48/45 responses were measured at the start, peak, and end of the transmission season[200]. The authors demonstrated variable boosting of responses over the transmission season that was more evident for Pfs48/45 than Pfs230. Therefore, further studies are required to verify the magnitude of such boosting and whether this translates to enhanced TBA.

Moreover, investigations into the development and maintenance of NA-TBI in relation to gametocyte density and duration of gametocytaemia in prospective longitudinal cohorts will improve our understanding of the dynamics and longevity of such immune responses[200]. Ideally, this should be carried out using a diverse panel of gametocyte-specific antigens. This would allow the identification of antigens with a

possible association to recent exposure to gametocytes that could be incorporated into serodiagnostic tools to define and monitor changes in the infectious reservoir. Results from the immunoprofiling suggest that a subset of the gametocyte antigens, most notably PEB-P, as well as crude gametocyte extract could serve as markers of recent gametocyte exposure; their prognostic ability should be investigated further.

7.2.2 Improving protein expression

Variable success with producing full-length ectodomains corresponding to the identified sexual stage antigens resulted in the production of 16 out of the 47 antigens. Characterisation of the remaining 31 antigens may identify more TBV candidates. Exploration of different protein expression platforms and optimising antigen sequences to overcome the *P. falciparum* 'AT-bias' could enhance the production of recombinant protein. Various platforms not investigated in this study that have been used to produce recombinant sexual stage antigens successfully include the baculovirus[288,303,619], *Lactococcus lactis*[314,425], and algal[620] expression systems. Where full-length protein production remains a challenge, protein structures of these antigens could be analysed *in silico* using epitope prediction software to identify potential epitopes or domains which may be more amenable to expression. The identification of critical domains of Pfs230 (region C[283,289]) and Pfs48/45 (fragment 10C[314,621]) capable of inducing potent TBA supports the use of fragments rather than full-length protein where size or complex tertiary structure impedes protein production.

7.2.3 Improving immunogenicity and efficacy (TBA)

Analysis of post-vaccination antibody titres after mice were vaccinated with the gametocyte proteins indicated that the majority of antigens were modestly immunogenic. Antibody titre has proven a useful correlate of TBV efficacy, and the low titres observed in this study for Pfs230 explain the modest TBA observed. This could also be true for the other gametocyte antigens, particularly CPP4, PSOP25 (both variants), MDV1 and G377B PfKE04 variant. Immunogenicity could be improved by optimising antigen concentrations, using different adjuvants[622], using other antigen delivery platforms such as viral vectors[289,584,595–597], or optimising the vaccination schedule[593,594] which could in turn increase the TBA observed. In a comparative assessment of 11 adjuvants in a mouse model of infection, Milicic *et al*. (2017) identified two adjuvants, Abisco®-100 and CoVaccineHT™ that were associated with

potentiated vaccine-induced responses and enhanced efficacy[622]. The ability of these adjuvants to improve the immunogenicity of the antigens studied in this work could be explored.

Other approaches could involve conjugating the proteins to immunogenic molecules such as *Pseudomonas aeruginosa* exoprotein A (EPA)[623,624] or outer membrane protein complex (OMPC) of *Neisseria meningitidis* serogroup B[625]. Alternatively, the use of viral vectors for antigen delivery rather than protein-in-adjuvant formulations could enhance both immunogenicity and potential longevity of the generated response. The ChAd63-MVA viral vector platform has been used successfully in heterologous prime-boost regimens for pre-erythrocytic[248,251,252], asexual[191,507], and sexual stage antigens[289], resulting in the induction of both antibody and cellular responses. Importantly, Kapulu *et al*. (2015), using the ChAd63-MVA platform for vaccine delivery in a rodent model, demonstrated induction of responses to Pfs230, Pfs48/45 and AnAPN1 that were sustained for 350 days post-vaccination[289] without the need for a booster dose. The potential for this approach to induce long-lasting responses to sexual stage antigens should be explored further in clinical trials.

7.2.4 Testing the P. falciparum orthologs of promising P. berghei candidates

Evaluation of gamete and ookinete antigens was based on a rodent model of malaria infection owing to the challenges of producing sufficient quantities of *P. falciparum* gametes and ookinetes *in vitro* for functional work. Advances in culture techniques [399,400] are providing solutions to circumvent this. In the future, it will be possible to use cultured *P. falciparum* gametes and ookinetes for TB assays such as the ookinete conversion assay. Alternatively, a rodent model could still be used but would require that mice be challenged with a chimeric *P. berghei* parasite that expresses the *P. falciparum* protein under investigation. Chimeric *P. berghei* parasites have been developed that express pre-erythrocytic[252,329] and sexual stage antigens[289,313,595,626] allowing the evaluation of *P. falciparum*-based vaccines in mouse vaccine efficacy studies.

Evaluation of TBA of the *P. falciparum* orthologs of the promising *P. berghei* candidates can also be achieved by SMFA analysis where cultured gametes and ookinetes are not required. Confirmation of the TBA seen in this study with sera

against the rodent proteins SOAP and PBCPP2 in *P. falciparum* will be of utmost importance if they are to be prioritised as TBV candidates. As PBCPP2 is an uncharacterised conserved *Plasmodium* protein, delineation of its structure and function could improve our understanding of sexual stage biology and provide insights on how to enhance its immunogenicity and efficacy. Moreover, the identification of orthologs in other *Plasmodium* species, e.g. *P. vivax,* may lead to the identification of conserved regions that could be targeted in a pan-species TBV.

7.2.5 Exploring the efficacy of multi-stage vaccine combinations

There is increasing support and evidence for the design of malaria combination vaccine that encompasses targets spanning various developmental stages of the parasite. Future work could also evaluate the possibility of combining promising antigens identified from this study with pre-erythrocytic or asexual stage targets. The synergy between pre-erythrocytic, asexual and sexual stage antigens co-administered in vaccines has been demonstrated to reduce both levels of infection and malaria transmission[313,316], highlighting a promising avenue for antimalarial vaccine development. Indeed, a study by Brod *et al*. (2018) showed synergy between the leading pre-erythrocytic vaccine candidate RTS,S/AS01 and the leading TBV candidate Pfs25 in mice[627]. Future studies should investigate more efficacious combinations and evaluate delivery platforms that ensure functional antigen-specific immune responses are generated to each vaccine component.

7.3 Final Remarks

This thesis aimed to provide an advancement of our understanding of NA-TBI and characterise a set of novel antigens as TBV candidates to inform TBV development efforts. This body of work has highlighted the potential for long-lived sexual stage responses and the influence of concurrent parasitaemia in the boosting of these responses. Additionally, indicators of recent gametocyte exposure such as age, parasitaemia, and malaria exposure were confirmed, and potential serological markers of recent exposure (PEB-P, CVMPPP, MDV1 and GE) identified. These could prove useful for evaluating the infectious reservoir. Finally, preliminary functional characterisation of the novel antigens identified the antigens SOAP and PBCPP2 as promising targets for further evaluation as vaccine candidates.

8. References

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9. Appendix

9.1 Appendix 1: Statement of Candidates Contribution

https://doi.org/10.7910/DVN/KXGFDI

9.2 Appendix 2: Supplementary information for the systematic review and meta-analysis of antibody responses to gametocyte antigens in African populations

Supplementary figures and tables for Chapter 2 are provided via the link below:

https://doi.org/10.7910/DVN/KXGFDI

9.3 Appendix 3: Supplementary information for the longitudinal analysis of gametocyte carriage (KMLRC cohort)

Supplementary tables for Chapter 3 are provided via the link below:

https://doi.org/10.7910/DVN/KXGFDI

9.4 Appendix 4: Reagents

A list of all commercially available reagents is available from the link below:

https://doi.org/10.7910/DVN/KXGFDI

9.5 Appendix 5: Buffers and Solutions

The buffers and solutions used for the laboratory assays are provided via the link below:

https://doi.org/10.7910/DVN/KXGFDI

9.6 Appendix 6: Vector Maps

The maps of the commercial vectors used for the cloning and expression are provided via the link below:

https://doi.org/10.7910/DVN/KXGFDI

9.7 Appendix 7: Recombinant protein production

A list of all primers prepared and used for in this work is provided in the link below:

https://doi.org/10.7910/DVN/KXGFDI

A summary of the sequence comparison between 3D7 and PfKE04 for the gametocyte, gamete and ookinete antigens is provided here:

https://doi.org/10.7910/DVN/KXGFDI

A description of the plasmid constructs prepared for recombinant protein production is provided in the link below:

https://doi.org/10.7910/DVN/KXGFDI

Sequences of the recombinant plasmids generated are provided in the link below:

https://doi.org/10.7910/DVN/KXGFDI

A summary of the mass spectrometry analysis of successfully produced recombinant protein is provided here:

https://doi.org/10.7910/DVN/KXGFDI

9.8 Appendix 8: ELISA optimisation for immunoprofiling of the gametocyte antigens

Graphs showing the ELISA optimisation for the immunoprofiling of the gametocyte antigens are provided via the link below:

https://doi.org/10.7910/DVN/KXGFDI

9.9 Appendix 9: Supplementary information for the immunoprofiling analysis

Supplementary information for the immunoprofiling analysis is provided via the link below:

https://doi.org/10.7910/DVN/KXGFDI

9.10 Appendix 10: Supplementary information for the functional characterisation of antibodies to the sexual stage antigens

Supplementary tables and graphs for Chapter 6 are provided via the link below:

https://doi.org/10.7910/DVN/KXGFDI